

FINAL REPORT

Bacterial and Benthic Community Response to Inorganic and Organic Sediment Amendments

SERDP Project ER-1551

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Table of Contents

List of Tables	iv
List of Figures.....	v
Acronyms.....	vii
Acknowledgements	ix
Executive Summary	x
Chapter 1: Ecotoxicological Response of Marine Organisms to Inorganic and Organic Sediment Amendments in Laboratory Exposure.....	1
1.1. Abstract	1
1.2. Objective	1
1.3. Technical Approach	3
1.4. Exposure Series #1: Toxicity in Uncontaminated Sediment	4
1.4.1. Amendments Used	4
1.4.2. Sediment Preparation	4
1.4.3. Toxicity Exposures	5
1.5. Exposure Series #2; Dose-Response Experiments with Chitin and Acetate	7
1.6. Exposure Series #3: <i>N. arenaceodentata</i> Exposures with Field-Contaminated Sediments	7
1.7. Water and Sediment Quality Measurements.....	7
1.8. Sediment, Pore water, and Tissue Sampling and Analysis	7
1.9. Data Analysis	8
1.10. Results.....	8
1.10.1. Exposure Series #1: Toxicity in Uncontaminated Sediment	8
1.10.2. Effects on Water Quality	9
1.10.3. Exposure Series #2: Dose-Response Chitin and Acetate Experiments	11
1.10.4. Exposure Series #3: <i>N. arenaceodentata</i> Toxicity and Bioaccumulation in Field-Contaminated Sediments Treated with Select Amendments.	14
1.11. Discussion	16
1.11.1. Lack of Toxicity Associated with Apatite and Organoclay	16
1.11.2. Water Quality Related Effects with Chitin and Acetate	17
1.11.3. Ammonia-Induced Toxicity in Chitin Treatments.....	17
1.11.4. Dissolved Oxygen Related Toxicity in Acetate Treatments.....	18
1.11.5. Polychaete Toxicity and Bioaccumulation in Amended Mare Island Sediment	19

1.12.	Benefits	21
1.13.	Literature Cited	21
Chapter 2: Bacterial Community Response to Inorganic and Organic Amendments in Marine Sediments		25
2.1.	Abstract	25
2.2.	Objective	25
2.3.	Technical Approach	26
2.4.	Experimental Design	26
2.4.1.	Sample Collection for Microbiology Studies	27
2.4.2.	Most Probable Number (MPN) Analysis	27
2.4.3.	Cell Number Determination by Epifluorescence Microscopy	27
2.4.4.	DNA Extraction, PCR and DGGE	28
2.4.5.	DGGE Band Sequencing and Phylogenetic Analysis	28
2.4.6.	X-Ray Absorption Spectroscopy	28
2.5.	Results	29
2.5.1.	Exposure Series #1; Stimulation of Bacterial Growth and Microbial Biomass	29
2.5.2.	Exposure Series #2: Microbial Response in the Dose-Response Chitin and Acetate Toxicity Experiments	30
2.5.3.	Exposure Series #3. Microbial Response in <i>N. arenaceodentata</i> Exposures with Field-Contaminated Sediments	30
2.5.4.	Microbial Community in Overlying Waters	31
2.5.5.	Bacterial Communities in Sediments vs. Overlying Water	33
2.5.6.	MPN Analysis	34
2.5.7.	XAS Analysis in Contaminated Sediments	35
2.6.	Discussion	38
2.6.1.	Effects of Inorganic Amendments on Bacterial Communities	38
2.6.2.	Effects of Organic Amendments on Bacterial Communities	39
2.6.3.	DGGE Analysis vs. MPN	40
2.6.4.	XAS Analysis	41
2.6.5.	Microbiology, Water Quality and Toxicology	42
2.7.	Benefits	42
2.8.	Literature Cited	43
List of Scientific/Technical Publications		47

List of Tables

Table 1-1. Overview of amendments used and their concentrations added to sediment in three different series of experiments.	3
Table 1-2. Characteristics of unamended test sediments. ERM= Effects range medium (Long et al. 1995). WQC= US EPA Water Quality Criterion for Saltwater. No value indicates parameter not measured. Reliable Detection Limit for bulk sediment metals analyzed using XRF is 50 µg/g dry weight.	5
Table 1-3. Summary of toxicity testing results from initial exposures involving reference site sediments (SD Control) amended with various materials either contained in reactive core mats or mixed loosely in the sediments. Values are means (\pm 1 s.d.). N=5.	9
Table 1-4. Ammonia and dissolved oxygen concentration for select experiments conducted in Exposure Series #1.	10
Table 1-5. Summary of no-observable-effects (NOEC) and lowest-observable-effects (LOEC) concentrations as well as median lethal concentrations of acetate and chitin to amphipods (<i>E. estuarius</i>) in multi-concentration exposures with these amendments in San Diego (SD) Bay sediment.	12
Table 2-1. Linear combination fitting of the samples from above as percent contribution for the mineral components. Hopeite is an independent component. Zn hydroxide-like phases falls into Zn hydroxide, Zn carbonate, and Zn LDH silicate. Sorbed Zn falls into Zn sorbed to ferrihydrite and gahnite. The R-factor is the error associated with the fits. ...	36
Table 2-2. Linear combination fitting of spiked (Cu, Zn, Cr) fine-grained sediments (Mare Island Naval Shipyard) with no amendments, 0.5% chitin amendment, 5% apatite amendment, and mixtures of chitin and apatite (0.5% and 5% respectively) amendments. These are the percent contributions for the mineral components. Hopeite is an independent component. Zn hydroxide-like phases falls into Zn hydroxide, Zn carbonate, and Zn LDH silicate. Sorbed Zn falls into Zn sorbed to ferrihydrite and gahnite. The R-factor is the error associated with the fits.	37

List of Figures

Figure 1-1. Relationship between sediment chitin concentration and unionized ammonia concentration measured in the overlying water following 10-day toxicity exposures with amphipods (<i>E. estuarius</i>).	11
Figure 1-2. Relationship between unionized ammonia concentration measured in overlying water and amphipod (<i>Eohaustorius estuarius</i>) survival following 10-day multi-concentration chitin exposure conducted in San Diego Bay sediment. Percentages refer to chitin sediment concentration (dry wt.).	12
Figure 1-3. Dissolved oxygen concentration (mg/L) in overlying water during multi-concentration acetate experiment conducted with amphipods (<i>E. estuarius</i>) over a 10-day exposure at 15 (top) and 20 °C (bottom). Amphipods were added on Exposure Day 0. N=3 replicates per measurement.	13
Figure 1-4. Survival (top) and final mean biomass (bottom) following 28-day polychaete (<i>N. arenaceodentata</i>) exposures using contaminated sediments from Mare Island Naval Shipyard (MI) and various combinations of apatite (Ap) and/or chitin (Ch). N=5.	15
Figure 1-5. Polychaete (<i>N. arenaceodentata</i>) whole body tissue concentrations for metals following 28-day exposures to sediment from Mare Island Naval Shipyard (MI) and various combinations of apatite (Ap) and/or chitin (Ch). N=3 replicates of 5 specimens each. Letters indicate statistical differences among treatments from pairwise comparison tests (Tukey's, $\alpha=0.05$).	16
Figure 2-1. Microbial biomass during the amendment experiments. A, bacterial cell counts of overlying water. T0, control on day 0 and a-j, cell counts on day 28: a, control; b, mat only; c, mat+apatite; d, mat+apatite+organoclay; e, apatite only; f, acetate only (1:10 dilution); g, chitin only; h, apatite+chitin; i, organoclay only; j, apatite+organoclay. B, epifluorescence microscopic observation of planktonic bacteria with amendments of acetate, chitin, apatite and chitin on day 10 and day 28. Other amendment treatments showed similar results as control. C, bacterial cell counts for dose-response experiments. Ac, acetate; Ch, chitin. *, significant difference compared to the control (unpaired t-test, $p < 0.05$)	30
Figure 2-2. Cell density, $1 \times 10^6/\text{mL}$, of bacteria in the overlying water column in course-grained and fine- grained sediments. Sediments were spiked with Cu, Zn, and Cr. Samples represent the control; CUU= Course- grained, Unamended, Un-spiked; CUS= Course- grained, Unamended, Spiked; CCS= Course- grained, Chitin amended, Spiked; CACS= Course- grained, Apatite + Chitin amended, Spiked; FUU= Fine-grained, Unamended, Un-spiked; FCU= Fine-grained, Chitin amended, Un-spiked; FACU= Fine-grained, Apatite + Chitin amended, Un-spiked; FUS= Fine-grained, Unamended, Spiked; FCS= Fine-grained, Chitin amended, Spiked; FACS= Fine-grained, Apatite + Chitin amended, Spiked.	31
Figure 2-3. DGGE fingerprints of bacterial community from overlying waters on day 28. Labels a-j were the same as Figure 2-1. Bands 1-20 were selected and excised for sequencing.....	32

Figure 2-4. Phylogenetic analysis of DGGE band sequences obtained from Figure 2-3. Most closely related representatives from GenBank were included. Bootstrap values were calculated based on 1000 resampling datasets. For clarity, only bootstrap values relevant to the interpretation of groupings were shown. Scale bar indicated the number of substitutions per site.	33
Figure 2-5. DGGE fingerprints of overlying waters and sediment bacterial communities exposed to acetate, chitin, and apatite+chitin collected on day 28. Amendments: f, acetate only; g, chitin only; h, apatite+chitin. Water samples (W) and three layers (T, top; M, middle and b, bottom) of sediments were included in the analysis. Bands 21-47 were excised for sequencing.	34
Figure 2-6. Phylogenetic analysis of DGGE band sequences obtained from Figure 2-5 and mostly closely related representatives from GenBank. Bootstrap values were calculated based on 1000 resampling datasets. For clarity, only bootstrap values relevant to the interpretation of groupings were shown. Scale bar indicated the number of substitutions per site.....	35
Figure 2-7. Example of XANES of Mare Island 3 samples over time post apatite amendment addition and reference spectra of primary components for linear combination fitting.....	36
Figure 2-8. Data from Table 2-2, represented graphically. Fine- grained sediments (MINSY) were spiked with additional Cu, Zn, and Cr. A: Unamended, control; B: Chitin (0.5%) amendment; C: Apatite (5%) amendment; D: Chitin (0.5%) and apatite (5%) amendment.	38
Figure 2-9. Bacteria plated unto medium containing insoluble apatite. Zones of clearing indicate phosphate-solubilizing bacteria (PSB).	41

Acronyms

As	Arsenic
ASTM	American Society for Testing and Materials
BOD	Biological Oxygen Demand
°C	Degrees Centigrade
Cd	Cadmium
cm	Centimeter
Cr	Chromium
Cu	Copper
DGGE	Denaturant Gradient Gel Electroporesis
D.O.	Dissolved Oxygen
EC50	Median Effects Concentration
Fe(II)	Iron 2+
g	Gram
H ₂ O	Water
HS ⁻	Hydrogen Sulfide
ICP-MS	Inductively-Coupled Plasma Mass Spectrometry
L	Liter
LC50	Median Lethal Concentration
LDH	Layered Double Hydroxides
LOEC	Lowest-Observable-Effects Concentration
mg	Milligram
MI	Mare Island
MINSY	Mare Island Naval Shipyard
μm	Micrometer (micron)
MPN	Most Probable Number
NAG	N-acetylglucosamine
Ni	Nickel
NOEC	No-Observable-Effects-Concentration
PAH	Polycyclic Aromatic Hydrocarbons
Pb	Lead
PCB	Polychlorinated Biphenyl
PCR	Polymerase Chain Reaction
PPM	Parts per Million
PSB	Phosphate-Solubilizing Bacteria

PSU	Practical Salinity Unit
RPM	Rotations per Minute
SD	San Diego
s.d.	Standard Deviation
SERDP	Strategic Environmental Research and Development Program
SPAWAR	Space and Naval Warfare Systems Command
SSC Pacific	SPAWAR Systems Center Pacific
SRB	Sulfate-Reducing Bacteria
TOC	Total Organic Carbon
USC	University of Southern California
USEPA	United States Environmental Protection Agency
Wt.	Weight
XANES	X-ray Absorption Near Edge Structure
XAS	X-ray Absorption Spectroscopy
XRF	X-Ray Fluorescence
YB	Yaquina Bay
Zn	Zinc

Keywords

marine amphipods, *Eohaustorius estuarius*/ marine polychaetes, *Neanthes arenaceodentata*/ purple sea urchins, *Strongylocentrotus purpuratus*/ larval sheepshead minnow, *Cyprinodon variegatus*/ bacterial community /amendments /marine sediments /mesocosms /marine benthic community/ apatite/ organoclay/ chitin/ acetate

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Executive Summary

The goal of this project was to evaluate the toxicity of several benthic community surrogates exposed to sediments without and with amendments intended to remediate mixed heavy metal contamination. Based on literature review, we evaluated apatite, an inorganic calcium phosphate amendment; and additional organic components such as chitin and acetate. In the first year, geotextile mats containing apatite and the organoclay, bentonite, were incorporated into the toxicity examinations in non-contaminated sediments. We examined all amendments singly and in combination for their potential ecological impacts and for their capacity to sequester and immobilize metals.

To assess impacts to benthic communities, a suite of laboratory marine toxicity and bioaccumulation tests were used. The benthic community members were the marine amphipods (*Eohaustorius estuarius*), marine polychaetes (*Neanthes arenaceodentata*), and purple sea urchins (*Strongylocentrotus purpuratus*), as these species are commonly employed as surrogates for general benthic community health. Larval sheepshead minnows (*Cyprinodon variegatus*) are a fish species known to be associated with surficial sediments and represent potential impacts to vertebrates. These were evaluated in the first year project but subsequently dropped as part of SERDP discussions.

A combination of biological (standard bioassay and bacterial data) and chemical (mineralogical and elemental composition) measurements were employed to detect how metals are partitioned to assess metal bioavailability, immobilization, and sequestration. The microbiological benthic community was examined to determine how the bacteria potentially affected the bioassay data as well as to examine bacterial species prevalent under varying sediment amendments.

Laboratory toxicity testing used in this project successfully demonstrated that apatite, organoclay, and geotextile mats containing those materials, were non-toxic in sediments at expected field application concentrations. Exposures were conducted on 4 different species (encompassing 6 different endpoints) representing a range of organism classes, life histories, and feeding approaches. Increased lethality and even beneficial effects (e.g., enhanced growth), were observed with acetate and chitin. Further analysis demonstrated that the static nature of the bioassay exposures likely contributed towards some of the observed negative effects. For example, increased ammonia concentrations were associated with increased bacterial growth due to acetate and chitin. This observation of enhanced ammonia in standard toxicity tests has also been observed in the evaluation of other amendments such as coal fly ash used for the removal of polycyclic aromatic hydrocarbons (Burgess, et al, 2009). Nevertheless, questions still remain as to whether or not these kinds of effects are representative of what might occur in the field. Subsequent testing with decreased concentrations of chitin demonstrated that chitin may stimulate a smaller increase in bacterial numbers that can potentially increase the effectiveness of the apatite amendment for apatite sequestration of the heavy metal zinc.

In conclusion, the amendments apatite, chitin, and geotextiles containing apatite and organoclay (in suggested concentrations) are considered non-toxic to marine invertebrates in marine sediments. This project was innovative in that it examined both the macro and micro biological benthic community after amendment additions and it helped determine the amendment combinations with the least harmful effects on benthic communities.

Chapter 1: Ecotoxicological Response of Marine Organisms to Inorganic and Organic Sediment Amendments in Laboratory Exposure

Gunther Rosen, Yolanda Meriah Arias-Thode, and James Leather

1.1. Abstract

Experimental materials currently being investigated for use as amendments for the *in situ* remediation of contaminated sediments were assessed for their potential impacts on marine benthos. Laboratory toxicity tests involving several endpoints were conducted on sediments amended with apatite, organoclay, chitin, or acetate; with the polychaete *Neanthes arenaceodentata*, the amphipod *Eohaustorius estuarius*, and the sheepshead minnow *Cyprinodon variegatus*. Experimental geotextile mats housing apatite and organoclay were also assessed. The geotextile mats, apatite (5%), and organoclay (5%) did not result in effects on any of the test organisms. Chitin and acetate, however, repetitively induced effects on survival and/or growth. The effects associated with chitin and acetate were attributed to water quality changes in the exposure vessels (ammonia and dissolved oxygen concentration) that were a direct result of the microbial breakdown of the amendments. A sediment concentration of 0.5% chitin or acetate was subsequently determined to be free from water quality effects in control sediments. *N. arenaceodentata* growth was enhanced in the presence of chitin, which stimulated bacterial growth that likely provided an additional food source for the polychaete. Application of chitin (0.5%) resulted in a statistically significant reduction in *N. arenaceodentata* body burdens of 61, 29, and 54%, relative to unamended contaminated sediment, for Cu, Zn, and Cd, respectively. The studies suggest a probable lack of inherent toxicity of these materials on benthic or epibenthic organisms, as the effects are expected to be related to artifacts associated with laboratory tests. Assessment in field settings are needed to verify such conclusions.

1.2. Objective

The objective of this work was to assess the ecological impacts to benthic communities of technologies currently in field use at contaminated sediment including various amendments. The toxicity of several benthic community surrogates exposed to sediments without and with amendments intended to remediate mixed heavy metal contamination was evaluated. Heavy metal contamination is a problem in marine and fresh water environments worldwide as a result of various industrial activities. Aquatic sediments tend to be an efficient sink for cationic metals such as cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn). The mobile, soluble forms of these metals are generally considered bioavailable and potentially toxic, as they easily pass through cell walls and can bioaccumulate (Rainbow 1993), presenting potential for trophic transfer to higher level organisms. Particle-associated metals can also be problematic due to the tendency for many benthic invertebrates to uptake contaminants through ingestion of sediment particles (Lee et al. 2000a).

Remedies for situations in which unacceptable environmental risk has been established at sediment sites include *ex situ* approaches such as dredging (removal of the contaminated sediment from the site), and *in situ* approaches including monitored natural recovery, and passive or reactive capping (USEPA 2005). Passive capping is a relatively economical remedy that

consists of a covering or cap of clean, inert material (e.g., sand) on top of contaminated sediment to provide a physical barrier that reduces contaminant migration to subsequent deposited sediment and the overlying water column. Passive caps, however, do not prevent toxic contaminants from being released due to processes such as leaching and mechanical disturbance, and can lead to substantial alteration of the benthic community due to their required thickness (Knox et al. 2008). Reactive capping, in contrast, involves the use of capping materials that react with sediment contaminants to reduce their toxicity or bioavailability (Millward et al. 2005, Reible et al. 2006, Knox et al. 2008). Therefore, reactive caps potentially provide not only the physical barrier that passive caps do, but also permanent sequestration of sediment-associated contaminants through reactions with the materials.

A variety of materials show promise for enhancing sequestration of organic and metal contaminants in sediments. For example, activated carbon has been shown to be useful for reducing bioavailability of PCBs and PAHs (Millward et al. 2005; Cho et al., 2007; McLeod et al. 2007; Janssen et al. in press), while metal sequestration has been shown to be viable in freshwater and saltwater environments with natural materials such as apatite (rock phosphate; Ma et al. 1993, Knox et al. 2003, Melton and Gardner 2004, Knox et al. 2008). Apatites are capable of reacting with heavy metals through both surface sorption reactions and precipitation reactions (Fedoroff et al., 1999; Singh et al., 2001; Bailliez et al., 2004), which can form chemically stable and insoluble compounds, particularly at estuarine and saltwater pHs (Chen et al. 1997). Organoclays (e.g., bentonite) have also been shown to remove non-polar organics contaminants as well as metals from water (Alther 2002, Knox et al. 2008).

While some experimental amendments have been added to sediments to sorb, degrade, transform or immobilize toxins and metals, others are added to stimulate growth of indigenous microorganisms to contribute to these processes (Robinson-Lora and Brennan 2009). For example, marine bacteria degrade chitin and release organic acids such as acetate and nitrogen (Bassler et al. 1991), which likely serve as substrates for sulfate reducing bacteria, promoting the precipitation of metals as sulfides, rendering the metals non-bioavailable.

Regardless of the amendment composition, accurate methods for introducing reactive materials to contaminated sediments are still in development. Cho et al (2007) describe the use of a large-scale mixing device suitable for working on tidal mudflats, while Menzie (2009) is demonstrating the use of a low-impact agglomerate (Sedimite™) that delivers treatment materials from the water surface. Geotextiles are porous, synthetic fabrics that could enable the accurate placement of a thin layer of highly sorptive media (i.e., activated carbon, apatite, organoclays) in the form of reactive mats at sediment sites (McDonough et al. 2007). These reactive core mats, or reactive barriers, allow for the movement of water and gasses through them, and therefore may be effective in areas of upwelling currents. They also require less material to stabilize contaminants than conventional chemical batch treatment because only mobile pollutants are treated.

It is imperative that materials used for *in situ* remediation of contaminated sediments are not only effective, but also do not pose any additional risk to the benthic or overlying communities. For example, while Millward et al. (2005) observed significant sequestration of PCBs from sediments treated with activated carbon, they also reported reduced growth of infaunal polychaetes relative to unamended sediments. This paper focuses on the assessment of the potential for marine benthic community effects of several materials currently being considered

for use as components of reactive caps. A series of laboratory toxicity experiments with multiple species and endpoints, and a single bioaccumulation study were conducted. Because of their critical roles in these processes, bacterial communities from overlying water and sediments were also characterized from these bioassays, and are presented in greater detail in a companion paper (Kan et al., submitted in concert with this paper).

1.3. Technical Approach

Three series of exposures were conducted (Table 1-1). The first exposure series involved an initial toxicity assessment of a range of amendments to three different marine test species following addition to uncontaminated sediment. The second set of experiments addressed water quality-related toxicity that was observed in the first series of experiments for two of the amendments. The third series of experiments used the results of the first and second exposure series to fine tune amendment concentrations for the laboratory exposure approaches used, and assess any difference in toxicity and uptake from a contaminated field sediment.

Table 1-1. Overview of amendments used and their concentrations added to sediment in three different series of experiments.

		Amendment Concentration (% dry sed. wt.)		
		Exposure Series #		
Treatment ID	Treatment Description	1	2	3
YB, SD, or MI	Unamended sediment	-	-	-
Mat	Mat Only	-	-	-
Mat-Ap	Mat + Apatite	10	-	-
Mat-Ap-O	Mat + Apatite + Organoclay	5 + 5	-	-
Ap	Apatite	5	-	0.5
O	Organoclay	5	-	-
Ap-O	Apatite + Organoclay	2.5 + 2.5	-	-
Ac	Acetate	5	0.5, 1.0, 2.5, 5	-
Ch	Chitin	2.5	0.1, .5, 1, 2.5	0.5
Ch-Ap	Chitin + Apatite	2.5 + 2.5	-	5 + 0.5

1.4. Exposure Series #1: Toxicity in Uncontaminated Sediment

1.4.1. Amendments Used

Four different materials were investigated, either singly or in combination: Phosfil apatite (rock phosphate mined from North Carolina), PM-199 organoclay (a proprietary granular clay compound marketed by Cetco Remediation Technologies, Hoffman Estates, IL), chitin (from crab shells, practical grade, coarse flakes, Sigma Aldrich, Product #C9213, CAS#1398-61-4), and anhydrous sodium acetate (>99.9% solid, Sigma Aldrich, CAS#127-09-3). Amendments were either mixed directly into sediment (see Section 1.4.2, Sediment Preparation) or housed inside reactive core geotextile mat samples (Cetco) that were placed beneath a 3 cm layer of uncontaminated sandy sediment from San Diego Bay to simulate subsequent application of a thin layer cap.

Amendment concentrations were selected based on the range used in recent laboratory and field studies for various types of amendments (Ma et al. 1995, Millward et al. 2005, Cho et al. 2007) and are shown in Table 1-1. The reactive mats were similar to those used by McDonough et al (2007), but were fabricated into circular shapes with a 3" diameter to accommodate the toxicity exposure chambers. The mat core was made from a high-loft polypropylene fiber that was needle-punched into a polypropylene woven geotextile. The high loft fibers had an opening size of 20 mesh (0.85 mm). The top of the mat was made from a non-woven polypropylene geotextile (pore size ~80 µm). Mats contained apatite and/or organoclay that reflected dry weight sediment concentrations shown in Table 1-1.

1.4.2. Sediment Preparation

Amendments were mixed into uncontaminated sediment collected from an uncontaminated site (SB2441; lat 32.69129, long -117.23803) located near the mouth of San Diego Bay (SD), CA. This location has been used previously as a reference site for ecological risk assessments (SCCWRP and SSC San Diego 2002). Physico-chemical characteristics of this sediment, and bulk metal concentrations are shown in Table 1-2. Sediment was pressed by hand through a 2 mm sieve to remove indigenous organisms and large particles prior to use. Sediment was stored at 4 °C for 3 days until use.

For the loosely mixed amendments, the appropriate amounts of sediment and amendment were added to 1 gallon glass jars, and initially mixed with an impeller mixer attached to a drill motor for 30 minutes. Following the initial mixing period, all jars were placed on a roll jar mill (US Stoneware, East Palestine, OH) for 48 hours for further homogenization and equilibration. Amended sediments were then added to pre-cleaned 1L glass mason jars, which served as the toxicity exposure vessels.

The reactive core mats were leached in uncontaminated flowing filtered seawater for 24 hours prior to addition to exposure jars. Mats were placed on the bottom of the jars with the nonwoven side up. This was followed by the addition of ~3 cm of SD control sediment. This exposure approach provided a suitable substrate for the test organisms (both toxicity and microbiological) that was intended to simulate either exposure following sediment deposition at a field site or conditions following placement of a thin layer cap on top of the reactive mat in the field.

Table 1-2. Characteristics of unamended test sediments. ERM= Effects range medium (Long et al. 1995). WQC= US EPA Water Quality Criterion for Saltwater. No value indicates parameter not measured. Reliable Detection Limit for bulk sediment metals analyzed using XRF is 50 µg/g dry weight.

	Yaquina Bay (YB)	San Diego Bay (SD)	Mare Island (MI)		ERM	WQC
	Bulk Sediment	Bulk Sediment	Bulk Sediment	Pore water		
	(µg/g)	(µg/g)	(µg/g)	(µg/L)	(µg/g)	(µg/L)
%silt/clay	2.5	18.5	7.9			
TOC (%)	0.06	0.90	0.77			
Cu		<50	<50	14	270	4.8/3.1
Zn		70	154	456	410	90/81
Cr		<50	1735	3.0	370	1100/50
As		<50	<50	59	70	69/36
Cd		<50	<50	2.6	9.6	40/8.8
Pb		<50	<50	ND	218	210/8.1
Ni		<50	160	46	52	74/8.2

1.4.3. Toxicity Exposures

Several lethal and sublethal standard and commonly used marine toxicity endpoints were used, representing different trophic levels and potential routes of contaminant exposure: 28-day polychaete (*Neanthes arenaceodentata*) survival and growth (ASTM 2000); 10-day amphipod (*Eohaustorius estuarius*) survival (USEPA 1994a); and 7-day larval sheepshead minnow (*Cyprinodon variegatus*) survival and growth (USEPA 1994b).

For each treatment, 150 g of sediment (a depth of approximately 3 cm) was added to each of 5 replicate beakers. A sixth replicate, also including organisms, was used for daily water quality (including pore water at test termination) measurements. Approximately 750 ml of uncontaminated, filtered (0.45 µm) natural seawater collected from near the mouth of San Diego Bay was added to each jar, followed by a 3 day equilibration period prior to addition of test organisms. All beakers were gently and continuously aerated at a rate of ~100 bubbles/minute.

Two negative controls were used for each test type, one consisting of unamended San Diego Bay sediment (SD Control), and the other consisting of uncontaminated sandy sediment collected from the amphipod collection site (Yaquina Bay, OR, referred to as YB Control). The latter was used to verify test acceptability (i.e., test organism health), while the former was used to assess any inherent toxicity associated with the San Diego Bay sediment, as well as to make statistical comparisons to the amended sediment treatments.

All exposures were conducted at a salinity of 30 psu, using 0.45- μ m filtered natural seawater collected on an incoming high tide at the SPAWAR Systems Center (SSC) Pacific bioassay laboratory, which is located near the mouth of San Diego Bay, CA.

Aqueous only reference toxicant tests using either copper or cadmium were conducted alongside all exposures and compared to laboratory control charts for batch sensitivity assessment for all bioassays.

1.4.3.1. *Polychaete survival and growth*

N. arenaceodentata survival and growth were assessed in 28-day static-renewal exposures (ASTM 2000) at a temperature of 20 °C. Two-week old post emergent juveniles were obtained from lab cultures held by Dr. Don Reish (California State University Long Beach, Long Beach, CA) and acclimated to test conditions at SSC Pacific for 3-5 days prior to exposure. Approximately 80% of the exposure water was renewed with clean seawater 2 times per week. Worms inhabiting each replicate were fed 1 mL of a well-mixed solution consisting of 1 g ground Tetramin fish flake food and 100 mL uncontaminated seawater following each renewal (equivalent to 4 mg Tetramin/worm/week). At the end of the exposure, the entire contents of each beaker were gently sieved using a 1 mm stainless steel screen. Worms were assessed for survival, rinsed in deionized water, and placed into pre-weighed aluminum weigh pans for wet weight determination. The samples were then dried to a constant weight in a drying oven 60 °C (2 days) for growth or biomass determination based on dry weight.

1.4.3.2. *Amphipod survival*

E. estuarius survival exposures were conducted following standard protocols (USEPA 1994a) with 3-5 mm field collected amphipods (Yaquina Bay, OR). Amphipods were acclimated to test conditions over 5-7 days prior to exposure. These experiments were conducted at 15 °C, in the presence of constant light, and were held static for 10 days. Upon termination of the exposure, beaker contents were sieved using a 1 mm stainless steel screen, and surviving amphipods enumerated.

1.4.3.3. *Sheepshead minnow survival and growth*

C. variegatus experiments followed guidance for chronic survival and growth testing with this species for whole effluent toxicity (USEPA 1994b), with the exception that fish were exposed in sediment-water interface instead of water only exposures. Early life stages of *C. variegatus* are negatively buoyant, tolerant of low dissolved oxygen, and tend to dig into sediment to hide from predators or to seek refuge from particularly warm or cold water (Sakowicz 2003). Ten, 7-day old larvae were exposed in each replicate. Exposures were conducted at 20 °C. Fish were fed twice daily with freshly hatched *Artemia* nauplii. Fish were recovered from the exposure beaker by gently swirling the beaker contents and pouring over a 1 mm screen. Fish were then rinsed in clean seawater and assessed for survival, and rapidly euthanized by severing the spinal cord with a scalpel. They were subsequently rinsed in deionized water to remove salts and placed in pre-weighed aluminum weigh boats. Following drying for 24 h at 60 °C, fish were weighed to the nearest 0.00001 g on a Sartorius Model 1712 balance.

1.5. Exposure Series #2; Dose-Response Experiments with Chitin and Acetate

Due to issues observed with water quality (ammonia and dissolved oxygen [D.O.] for chitin and acetate, respectively) during initial toxicity experiments with chitin and acetate, a second set of experiments was conducted with *E. estuarius* only to confirm suspected causes of observed toxicity as well as optimize the exposure approach for subsequent experiments (e.g., Exposure Series #3).

Chitin and acetate were separately mixed directly into uncontaminated SD sediment in four different concentrations (Table 1-1) using the same sediment preparation procedures used in Section 2.1. Chitin was tested at 15 °C only, while acetate experiments were conducted at both 15 and 20 °C. *E. estuarius* was selected for these experiments because of the test method's static nature, representing worst case, and the relatively high sensitivity of this species to ammonia.

1.6. Exposure Series #3: *N. arenaceodentata* Exposures with Field-Contaminated Sediments

In order to assess whether or not the amendments evaluated in clean sediment would also be non-toxic in field sediments contaminated with metals, similar exposures were conducted using a field-contaminated sediment. Course and fine-grained sediments from Yaquina Bay, Oregon and Mare Island Naval Shipyard (MINSY) were evaluated. Because these sediments were aged, additional spikes of Cu, Zn, and Cr were incorporated with the goal of metal concentrations in the overlying water of Cu= 0.25 ppm; and Zn and Cr=1.0 ppm. These concentrations had to assume no binding as TOC of coarse sand < 0.1%.

Sediment was collected from Green Sands Beach at Mare Island Naval Shipyard (lat 38.086, long -122.255) and selected based on historically high bulk sediment metal concentrations (Table 1-2). Exposures were conducted as described for other experiments with *N. arenaceodentata*, differing only in that following the 28-day experiments, surviving worms were assayed for whole body metal concentration. This involved purging the guts of the exposed worms in clean seawater overnight, followed by wet weight determination. Worms were dried in 2 mL polypropylene microcentrifuge tubes to constant weight at 60 °C (2 days) in preparation for nitric acid digestion and subsequent analysis by inductively coupled plasma mass spectrometry (ICP-MS).

1.7. Water and Sediment Quality Measurements

Overlying water quality (pH, dissolved oxygen, salinity, temperature) was recorded daily in surrogate chambers for each bioassay using standard laboratory equipment. Ammonia was measured in both the overlying water and pore water at test initiation and test termination for each bioassay using an ammonia salicylate method (10031) with a Hach DR/2400 spectrophotometer. Pore water was collected from the test chambers by pouring off the overlying water of the surrogate beakers, and centrifuging a portion of the sediment at approximately 4000 RPM for 20 minutes. Unionized ammonia was calculated based on the pH, salinity, and temperature of the overlying water and pore water samples (USEPA 1989).

1.8. Sediment, Pore water, and Tissue Sampling and Analysis

Bulk sediment metals concentrations were measured using X-Ray Fluorescence (XRF) using EPA Method 6200. Samples were dried and 10g of dry sediment was analyzed by XRF for As, Cd, Cr, Cu, Ni, Pb, and Zn. Pore water metal concentrations were determined following

recommendations of Bufflap and Allen (1995). Following bioassays, one extra test chamber had overlying water siphoned off and was placed in an anaerobic chamber where the wet sediment was sealed in a centrifuge tube and then removed from the chamber and spun at 4000rpm for 30 minutes. Centrifuge tubes were returned to the anaerobic chamber where pore water was recovered and filtered at 0.45 μ before being acidified (below pH of 2) and analyzed by ICPMS for metals including As, Cd, Cr, Cu, Ni, Pb, and Zn.

After weighing, dried worms underwent nitric acid digestion and whole body metals concentrations were determined using ICP-MS following procedures described in Rosen et al. (2008).

1.9. Data Analysis

Comparisons between amendments and SD controls were made using t-tests, following arc-sine square root transformation of data associated with the specific endpoint. Where relevant, no-observable-effects-concentrations (NOEC) and lowest-observable-effects concentrations (LOEC), as well as median lethal (LC₅₀) or effects (EC₅₀) concentrations were calculated with the assistance of ToxCalc 5.0 (Tidepool Scientific). One-way analysis of variance ($\alpha=0.05$) was used to make statistical comparisons among treatment effects on polychaete body burden, with Tukey's test used for making pairwise comparisons.

1.10. Results

1.10.1. Exposure Series #1: Toxicity in Uncontaminated Sediment

Results from the three different toxicity tests for each amendment type or combination are summarized in Table 1-3. Control performance was high, ranging from 89-100% in both the YB and SD sediments for all endpoints. No significant effects were observed relative to the SD control for either the reactive mats by themselves or reactive mats containing apatite and/or organoclay. Similarly, loosely mixed apatite and organoclay resulted in no adverse effects whether added individually or in combination to sediment for any endpoint.

Statistically significant reductions in *E. estuarius* survival and *C. variegatus* growth were observed in the presence of chitin, regardless of whether exposed singly or in combination with apatite (Table 1-3), with the largest effects being observed for *E. estuarius* survival. Mean *C. variegatus* survival was also reduced in the presence of chitin, but this difference was not statistically significant.

In contrast, *N. arenaceodentata* biomass was significantly enhanced in the presence of chitin, by a factor of ~2, relative to the SD control (Table 1-3). Although not statistically significant, *N. arenaceodentata* biomass in the presence of acetate was higher than the SD control by a factor of ~1.5. Unlike organoclay or apatite-amended sediments, the chitin and acetate treatments rapidly induced prominent bacterial blooms, which resulted in marked coloration changes of the overlying water.

N. arenaceodentata survival was statistically lower only in the acetate treatment (Table 1-3). Survival in the acetate treatment, however, was rather variable among replicates, and individual beakers showing reduced survival corresponded with nearly depleted dissolved oxygen (D.O) concentration at times during the exposure.

Table 1-3. Summary of toxicity testing results from initial exposures involving reference site sediments (SD Control) amended with various materials either contained in reactive core mats or mixed loosely in the sediments. Values are means (\pm 1 s.d.). N=5.

	<i>E. estuarius</i>	<i>C. variegatus</i>		<i>N. arenaceodentata</i>	
Treatment	Survival (%)	Survival (%)	Growth (mg)	Survival (%)	Biomass (mg)
YB Control	96 (4.2)	98 (5.0)	0.777 (0.083)	96 (8.9)	24.3 (3.63)
SD Control	89 (9.6)	100 (0)	0.666 (0.086)	100 (0)	24.9 (4.86)
Mat	94 (4.2)	100 (0)	0.676 (0.180)	92 (11)	28.8 (5.99)
Mat-Ap	94 (8.2)	100 (0)	0.740 (0.040)	96 (8.9)	26.8 (4.35)
Mat-Ap-O	88 (7.6)	100 (0)	0.607 (0.152)	100 (0)	28.9 (0.74)
Ap	92 (5.7)	100 (0)	0.773 (0.105)	100 (0)	29.2 (2.67)
O	90 (9.4)	100 (0)	0.697 (0.068)	96 (8.9)	25.3 (3.35)
Ap-O	95 (3.5)	100 (0)	0.720 (0.058)	100 (0)	27.4 (4.38)
Ac	86 (7.5)	95 (5.8)	0.467 (0.054)*	50 (49)*	39.0 (7.88)
Ch	12 (8.4)*	65 (41)	0.407 (0.168)*	100 (0)	52.0 (12.9)*
Ch-Ap	27 (21.7)*	48 (55)	0.401 (0.066)*	100 (0)	47.9 (8.90)*

*Indicates statistically different from SD Control using unequal variance t-tests ($\alpha=0.05$).

1.10.2. Effects on Water Quality

Unionized ammonia concentrations in exposure beaker overlying water and pore water are summarized in Table 1-4. Ammonia concentration was relatively low and similar to the SD control treatment for most amendments, but was consistently elevated in chitin treatments, sometimes to concentrations that approached or exceeded known toxicity thresholds for the various endpoints (Table 1-4; USEPA 1989, Dillon et al. 1993, USEPA 1994, Kohn et al. 1994).

In contrast to chitin, acetate routinely resulted in lower ammonia concentrations than those measured in the SD control treatment (Table 1-4). The D.O. concentration in the presence of acetate, was also regularly lower than most other treatments during the first two weeks of the *N. arenaceodentata* exposure (Table 1-4), and dipped below that tolerated by the polychaetes on Day 4 of the exposure. When chambers were renewed on this day, dead polychaetes in the acetate treatment were observed on the sediment surface.

Differences in other physical parameters, including pH, temperature, and salinity, were negligible among treatments.

Table 1-4. Ammonia and dissolved oxygen concentration for select experiments conducted in Exposure Series #1.

Treatment	Unionized ammonia (mg/L NH ₃)					Diss. oxygen (mg/L)	
	<i>E. estuarius</i>		<i>N. arenaceodentata</i>		<i>C. variegatus</i>	<i>N.arenaceodenata</i>	
	Day 10		Day 28		Day 7	Min	Max
	OW	PW	OW	PW	OW	OW	OW
YB Control	ND	ND	ND	0.004	ND	6.6	7.9
SD Control	0.163	0.434	0.004	0.023	0.173	6.7	8.0
Mat	0.129	0.311	NM	NM	0.181	6.9	7.9
Mat-Ap	0.118	0.300	0.004	0.058	0.092	6.7	7.9
Mat-Ap-O	0.126	0.292	0.023	0.004	0.116	4.5	7.9
Ap	0.155	0.498	0.000	0.023	0.162	4.6	7.9
O	0.137	0.399	0.035	0.035	0.135	6.7	8.0
Ap-O	0.134	0.560	0.000	0.015	0.150	6.6	7.9
Ac	ND	0.155	0.104	0.123	ND	1.3	8.0
Ch	1.18	2.38	0.610	1.91	0.993	4.8	6.7
Ch-Ap	1.15	2.78	0.801	1.90	0.959	6.0	7.4
NOEC	0.8 ^a	0.8 ^a	0.68 ^c	0.68 ^c		1.5 ^c	1.5 ^c
LOEC			1.25 ^c	1.25 ^c			
LC50	2.49 ^d	2.49 ^d			2.717 ^d		

^aUSEPA (1994), for survival

^bKohn et al. (1994)

^cDillon et al. (1993). NH₃ NOEC=growth; LOEC=survival. D.O., NOEC= survival.

^dUSEPA (1989)

1.10.3. Exposure Series #2: Dose-Response Chitin and Acetate Experiments

1.10.3.1. Chitin Experiment

Ammonia concentration in the overlying water was positively correlated with increasing chitin concentration added to SD sediment (Figure 1-1; $r^2 = 0.909$). A dose-response was also observed with increasing chitin and corresponding ammonia concentrations, with partial mortality occurring at a chitin concentration of 1% and complete mortality of 2.5% in SD sediment (Figure 1-2). Toxicity metrics including NOEC, LOEC, and LC50s from the dose response experiment with chitin are shown in Table 1-5. Unionized ammonia concentrations in the overlying water exceeded the 0.8 mg/L NOEC for *E. estuarius* in the 2.5% treatment (0.905 mg/L), and approached the NOEC in the 1% treatment (0.645 mg/L). Although pore water concentrations were not measured in this experiment, unionized ammonia concentration in the pore water in Exposure Series #1 were 2 times that of the overlying water, suggesting that pore water concentrations in both of these treatments would have been toxic to the *E. estuarius*.

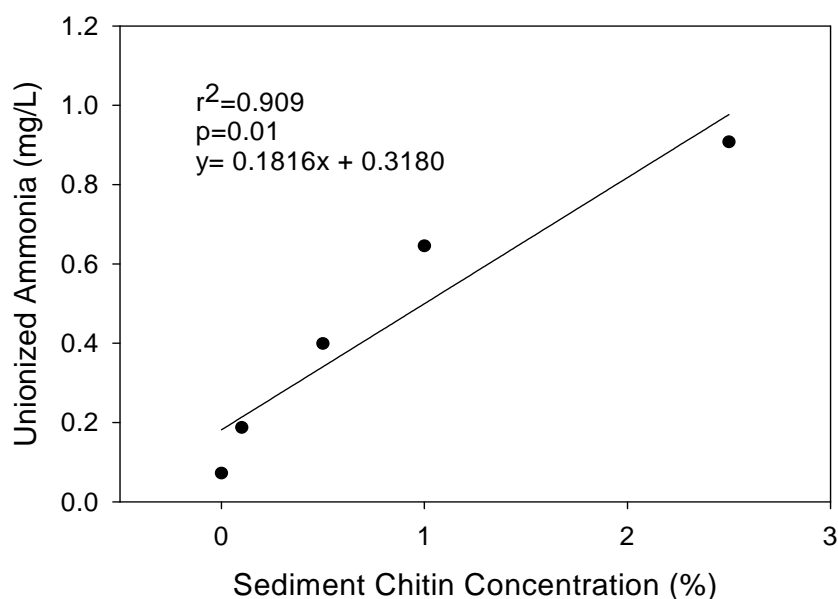


Figure 1-1. Relationship between sediment chitin concentration and unionized ammonia concentration measured in the overlying water following 10-day toxicity exposures with amphipods (*E. estuarius*).

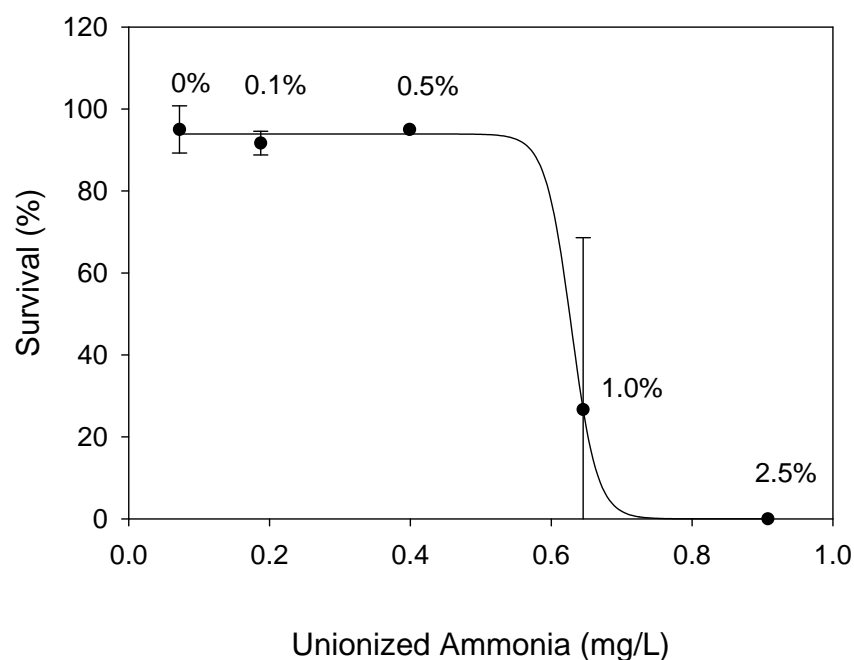


Figure 1-2. Relationship between unionized ammonia concentration measured in overlying water and amphipod (*Eohaustorius estuarius*) survival following 10-day multi-concentration chitin exposure conducted in San Diego Bay sediment. Percentages refer to chitin sediment concentration (dry wt.).

Table 1-5. Summary of no-observable-effects (NOEC) and lowest-observable-effects (LOEC) concentrations as well as median lethal concentrations of acetate and chitin to amphipods (*E. estuarius*) in multi-concentration exposures with these amendments in San Diego (SD) Bay sediment.

	NOEC	LOEC	LC50
Ac 15 °C (%)	5	>5.0	>5.0
Ac 20 °C (%)	0.5	1	0.86
Ch 15 °C (%)	0.5	1	0.92
Ch-generated Ammonia (mg/L) 15 °C	0.40	0.65	0.57

1.10.3.2. Acetate Experiments

Summary toxicity metrics (NOEC, LOEC, LC50) for the multi-concentration acetate experiments are shown in Table 1-5. The D.O. concentration remained within acceptable ranges

(>4 mg/L; USEPA 1994) in the 15 °C treatments through Day 7, but rapidly declined towards critical levels at all concentrations greater than 0.5% (Figure 1-3). In the 20 °C experiment, D.O. rapidly dropped to concentrations well below acceptable in the toxicity test prior to organism addition, recovered, and then steadily dropped towards anoxia by Day 7, where the D.O. remained for the 2.5 and 5% treatments. One replicate from the 1% treatment resulted in relatively high D.O. concentration after Day 7, while the other 2 replicates were 0 mg/L. The one replicate where concentrations remained within acceptable concentrations for *E. estuarius* resulted in 85% survival, while the other two replicates resulted in no surviving amphipods. Therefore, it was quite apparent that D.O. concentration directly affected survival at concentrations above 0.5% acetate.

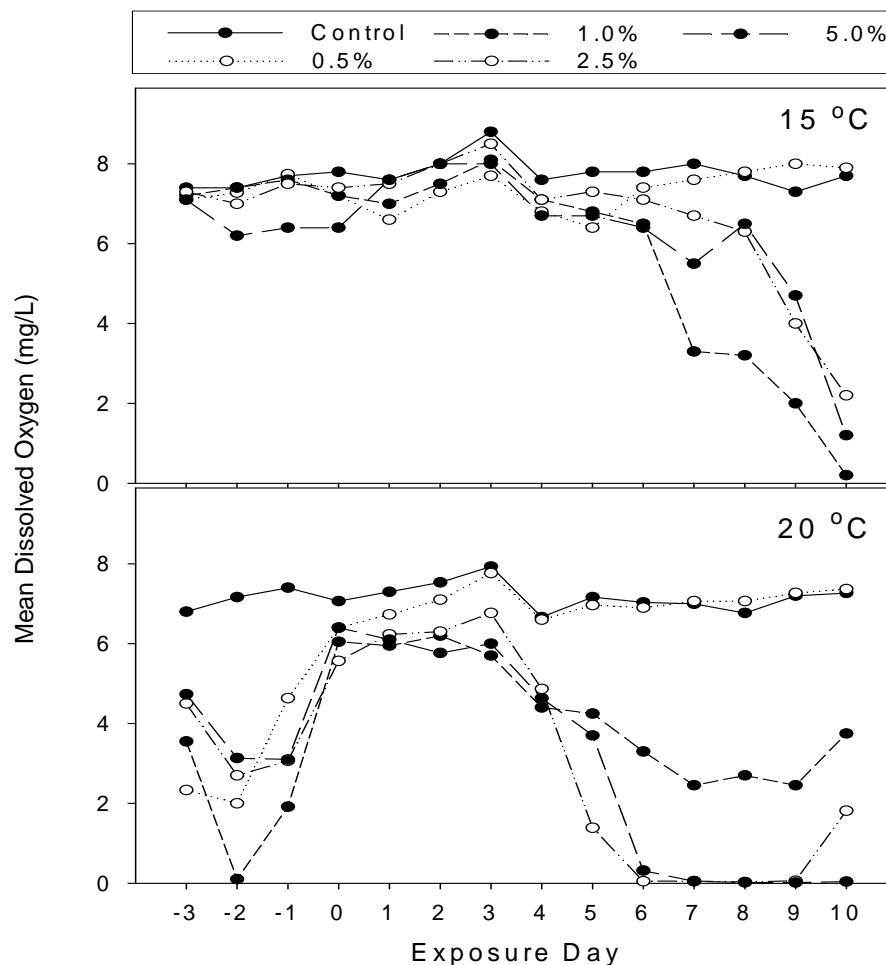


Figure 1-3. Dissolved oxygen concentration (mg/L) in overlying water during multi-concentration acetate experiment conducted with amphipods (*E. estuarius*) over a 10-day exposure at 15 (top) and 20 °C (bottom). Amphipods were added on Exposure Day 0. N=3 replicates per measurement.

1.10.4. Exposure Series #3: *N. arenaceodentata* Toxicity and Bioaccumulation in Field-Contaminated Sediments Treated with Select Amendments.

Survival of *N. arenaceodentata* was high in all treatments (range = 92-100%; Figure 1-4) and was not statistically lower for neither the MI sediment nor any amendment or amendment combination in MI sediment. Even at the reduced chitin concentration of 0.5%, however, *N. arenaceodentata* growth was enhanced by a factor of ~1.6 in the presence of chitin or chitin combined with apatite, which was statistically higher than unamended MI sediment (Figure 1-4). Visual examination of the overlying water indicated substantial bacteria growth in both treatments containing chitin.

N. arenaceodentata body burdens for Cu, Zn, Cd, and As were higher in MI sediment, sometimes substantially (e.g., factor of 6 for Cu), relative to the YB control sediment used in this experiment (Figure 1-5). Apatite did not result in reduced body burdens for any of the four metals relative to the unamended MI sediment. When MI sediment was amended with 0.5% chitin or 0.5% chitin combined with 5% apatite, however, body burdens were reduced by 61-63, 29-32, and 54-55%, for Cu, Zn, and Cd, respectively. The presence of chitin, however, resulted in a 57% increase in As concentrations, relative to unamended MI sediment (Figure 1-5).

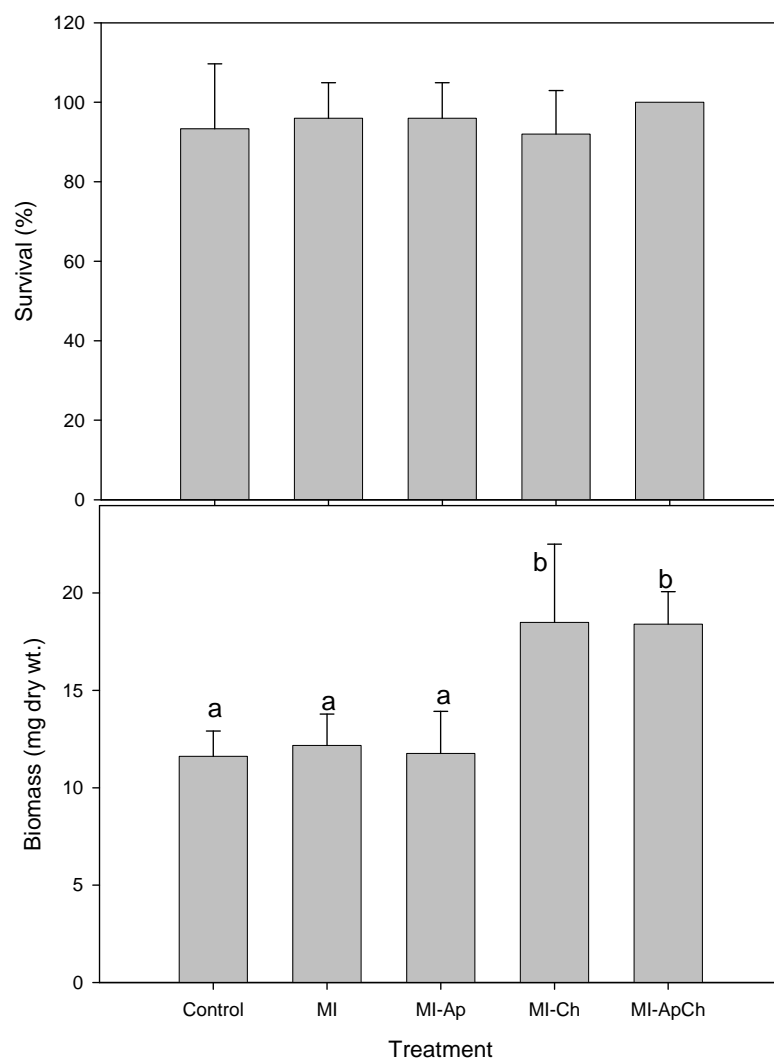


Figure 1-4. Survival (top) and final mean biomass (bottom) following 28-day polychaete (*N. arenaceodentata*) exposures using contaminated sediments from Mare Island Naval Shipyard (MI) and various combinations of apatite (Ap) and/or chitin (Ch). N=5.

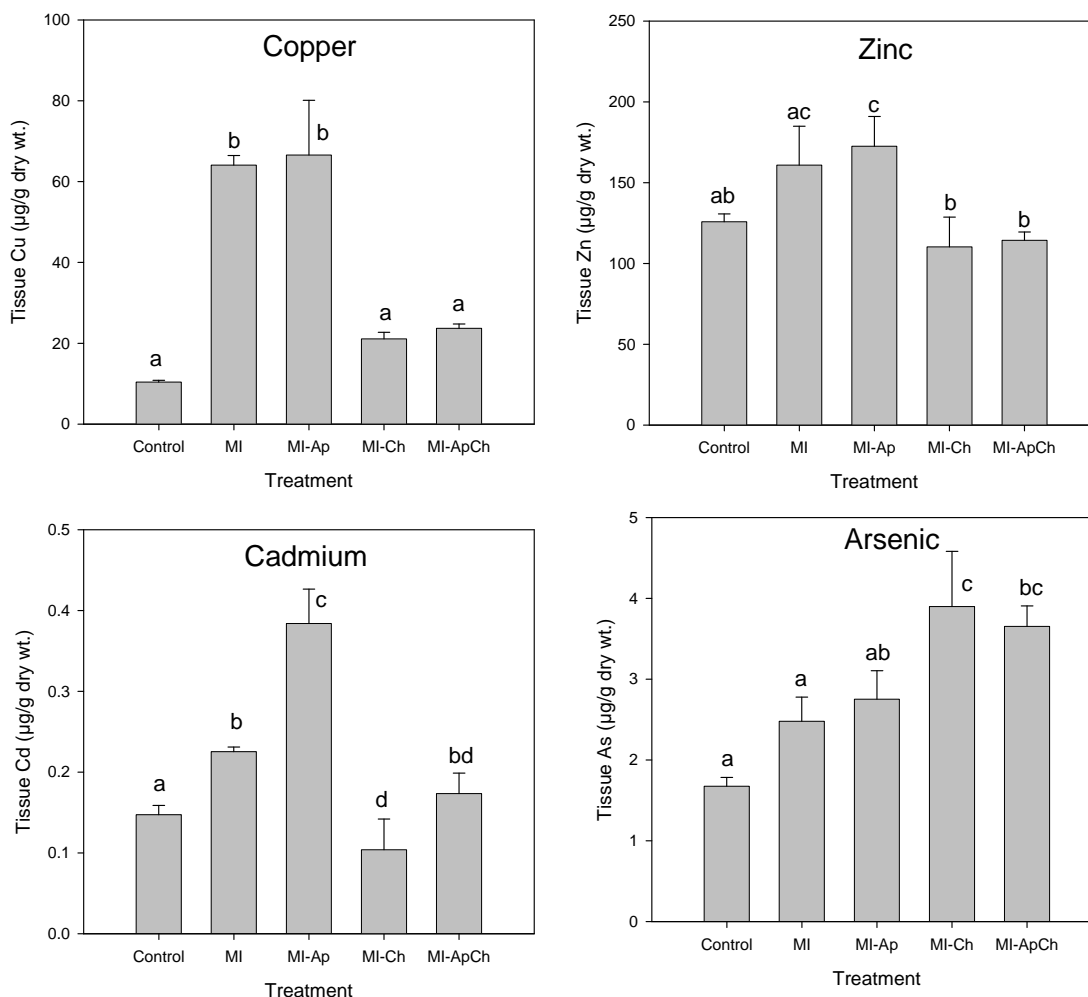


Figure 1-5. Polychaete (*N. arenaceodentata*) whole body tissue concentrations for metals following 28-day exposures to sediment from Mare Island Naval Shipyard (MI) and various combinations of apatite (Ap) and/or chitin (Ch). N=3 replicates of 5 specimens each. Letters indicate statistical differences among treatments from pairwise comparison tests (Tukey's, $\alpha=0.05$).

1.11. Discussion

1.11.1. Lack of Toxicity Associated with Apatite and Organoclay

Neither apatite nor organoclay resulted in statistically significant effects for any of the test endpoints evaluated, whether loosely mixed or contained in reactive core mats. Therefore, it is expected that these materials should not have negative impacts on natural benthic communities at the concentrations employed in this study. Lack of toxicity of North Carolina apatite and organoclay was also observed by Paller and Knox (in press) in laboratory exposures to fresh and brackish sediment dwelling invertebrates at similar concentrations. Rather, North Carolina apatite has been shown to very effectively sequester metals, particularly Cu and Zn, in both fresh and saltwater (Knox et al. 2008), reducing the bioavailability of potentially toxic metals. In addition, phosphate solubilizing bacteria have been shown to enhance the precipitation of metals

from solution by forming insoluble metal phosphates (Ayyakkannu and Chandramohan, 1971) resulting in permanent sequestration.

It should be noted that it is possible that the test organisms used in this study had relatively little contact with the contents of the reactive core mats due to their presence 3 cm below the uncontaminated sediment layer and the lack of upwelling conditions in the exposure vessels. *N. arenaceodentata*, however, were observed in some cases clinging to the exterior of the mats themselves upon recovery from the exposure, suggesting that there was no toxicity or avoidance response associated with the geotextiles. The presence of increased bacteria numbers in the overlying water in treatments containing loosely mixed apatite and organoclay relative to treatments where mats held the apatite and organoclay (Figure 2-1A) is suggestive of less interaction between the amendments and the sediment-water interface in the latter under this exposure system.

1.11.2. Water Quality Related Effects with Chitin and Acetate

Unlike with apatite and organoclay, lethal and sublethal effects were observed for some species in treatments where organic amendments (acetate or chitin) were present. Treatments in which effects were observed, however, were also associated with documented enhanced microbial activity (Figure 2-1A) that appears to be indirectly responsible for toxicity via a reduction in water quality (i.e., ammonia and D.O. concentration), as verified by subsequent dose-response experiments (Exposure Series #2).

1.11.3. Ammonia-Induced Toxicity in Chitin Treatments

Chitin treatments resulted in overlying and pore water ammonia concentrations substantially higher than any of the other treatments. Ammonia is a normal breakdown product of chitin (Campbell and Williams 1951, Bassler et al. 1991), a polysaccharide that is a food source for aerobic and some anaerobic bacteria (Osawa and Koga, 1995; Keyhani and Roseman, 1999). The breakdown of chitin is catalyzed by bacteria-produced enzymes (chitinases), which result in cleaving of the glycosidic bonds and conversion of chitin to simple sugars (such as acetate) and ammonia.

The dramatic increases in unionized (the fraction that is generally considered to be the most toxic) ammonia concentration observed in all exposures with chitin explain the observed effects on *E. estuarius* survival and likely contributed to or caused the reduced growth of *C. variegatus*. The elevated overlying water and pore water ammonia concentrations measured in the initial chitin experiments, which employed a chitin sediment concentration of 2.5%, were in excess of published thresholds for *E. estuarius* (USEPA 1994, Kohn et al. 1994). The subsequent dose-response chitin experiment conducted with *E. estuarius*, indicated that toxicity was correlated with the ammonia concentration, providing strong evidence that the observed toxicity was a result of the breakdown of chitin and not the chitin itself. However, it should be noted the 0.5% chitin NOEC derived in this study is specific to the unique physico-chemical characteristics of the SD sediment used and the laboratory exposure design employed in this study.

In addition to impacts on *E. estuarius* survival, reduced growth of *C. variegatus* for 2.5% chitin treatments corresponded with increased unionized ammonia concentrations (as high as 0.993 mg/L), but ammonia thresholds for *C. variegatus* growth are unknown. The reported unionized ammonia 96-h LC50 for this species is 2.72 mg/L (USEPA 1989), but it is likely that

growth effects occur at substantially lower concentrations. For comparison purposes, *Menidia beryllina* (inland silverside) growth is significantly reduced at concentrations 21 times lower than the 96 h LC50 (USEPA 1989). Applying such a conversion to *C. variegatus* would suggest that reduced growth would have occurred due to the ammonia concentrations (> 0.13 mg/L) observed in the overlying water. It is also likely that *C. variegatus* were exposed to a mix of overlying and pore water ammonia concentrations, thus resulting in an even greater exposure to ammonia than that reported here. The larvae of this species is negatively buoyant and is known to dig in surficial sediments (Sakowicz 2003), and was typically observed foraging at the sediment-water interface, stirring up sediment during the process.

The lack of ammonia-related effects on survival or growth of *N. arenaceodentata* could be partly due to the nature of the exposure regime (2x weekly renewals), which resulted in lower final ammonia concentrations as compared with the static *E. estuarius* exposures (Table 4). Ammonia concentrations associated with chitin were also only marginally in excess of published thresholds for these endpoints in the overlying water. Unlike the free-burrowing amphipods that are exposed directly to pore water, *N. arenaceodentata* were likely protected from direct pore water exposure by their mucoid tubes (Dillon et al. 1993).

Elevated ammonia concentration is not a unique finding in toxicity tests used to evaluate sediment amendments, and raises concerns with how future assessments of their biological effects should be conducted. Burgess et al (2009) reported that some types of coal fly ash increased both ammonia concentration and pH in the overlying water of toxicity test chambers to levels in excess of reported ammonia LC50s for the species that were exposed. Although the pH increases were not suspected to directly cause the observed toxicity to amphipods in their study, the increase in un-ionized ammonia concentration associated with the pH corresponded with mortality of marine invertebrates. Ammonia is a natural breakdown product of chitin, and therefore, would be expected to be present at elevated concentrations. Real world deployments might result in improved understanding of how the process might affect water quality and subsequent effects on the biota.

This study used practical grade chitin, which was selected due to its relative cost effectiveness. Organic matter associated with the relatively crude grade of product used could have contributed to the excess food supply. Therefore, investigation into similar materials such as fine grade chitin or chitosan (produced by deacetylation of chitin) is recommended. Mesocosm studies supporting realistic continuous replacement of overlying water or real world responses would likely not result in ammonia buildup to toxic concentrations.

1.11.4. Dissolved Oxygen Related Toxicity in Acetate Treatments

Acetate serves as a carbon source to stimulate indigenous bacterial communities that might be able to reduce metals and immobilize them from contaminated systems (Istok et al. 2004). It is also a product from the breakdown of chitin (Bassler et al. 1991), and was thus explored in this study as a means of stimulating the growth of sulfate-reducing bacteria, which could in turn reduce metal bioavailability by the creation of metal-sulfides. It is unlikely, however, that acetate would be used as an amendment for the remediation of contaminated sediments due to its tendency to readily dissolve upon exposure to seawater, as well as the cost effectiveness of chitin relative to acetate.

Acetate treatments resulted in reduced *N. arenaceodentata* survival in some beakers, and reduced *C. variegatus* growth. *N. arenaceodentata* mortality in Exposure Series #1 was attributed to a temporary, but sharp decline in D.O. concentration (to 1.3 mg/L) that was observed on Day 4 of the exposure in the surrogate chambers used for water quality measurements. Scheduled renewal of the overlying water on this day revealed deceased worms on the sediment surface. The recommended minimum D.O. concentration during these tests is 4 mg/L (ASTM 2000), while *N. arenaceodentata* survival was reduced at a D.O. concentration of 1.0 mg/L in 96 h aqueous exposures (Dillon et al. 1993). It is suspected that very high biological oxygen demand (BOD) associated with the utilization of acetate by indigenous bacteria as a food source, even in the presence of continuous aeration, resulted in D.O. concentrations below those required to maintain *N. arenaceodentata* survival.

High BOD and depressed D.O. concentrations in toxicity tests with rainbow trout from deicing products containing sodium acetate have been reported with lethality occurring at aqueous concentrations of 16.1 g/L (Bang and Johnston 1998). In this study, initial acetate concentrations in the water column from *C. variegatus* exposures would have achieved a maximum concentration of 6.6 g/L, but no mortality was observed.

The subsequent multi-concentration acetate experiment (Exposure Series #2) confirmed the incidence of reduced D.O. concentrations at acetate concentrations greater than 0.5%. As was observed in the initial experiments, within a treatment (i.e., 1% acetate), some replicates resulted in D.O. concentrations as low as 0 mg/L, while others were near saturation (~8 mg/L). Likewise, variability in *E. estuarius* survival within treatments was observed in the multi-concentration acetate experiment conducted at 15 °C, and is likely to be due to precipitous declines in D.O. in some replicates. The reliably low *E. estuarius* survival in the 20 °C multi-concentration acetate experiment is likely due to considerably lower D.O. concentrations measured at this temperature (where bacterial activity would be expected to be greater), which was generally observed for all replicates. The more extreme D.O. declines, particularly early on in the exposure, at the higher temperature are likely due to the increased metabolism rate and microbial activities of ambient bacterial groups (Vinolas et al. 2001). This helps explain why *N. arenaceodentata*, which is typically exposed at the higher temperature, suffered from mortality in the presence of acetate, while *E. estuarius* did not in Exposure Series #1.

In contrast to chitin, acetate routinely resulted in lower ammonia concentrations than those measured in the SD control treatment. In the parallel microbiology study, Kan et al. showed that acetate yielded twice as many bacteria as chitin in the overlying water collected from these experiments, which contained different bacterial groups belonging to Alphaproteobacteria (see Chapter 2).

1.11.5. Polychaete Toxicity and Bioaccumulation in Amended Mare Island Sediment

1.11.5.1. N. arenaceodentata increased growth

Individual food rations for these experiments were 4 mg ground Tetramin/worm/week, which was intended to be below what *N. arenaceodentata* can consume so as to maximize sensitivity of the toxicity tests (Bridges et al. 1997). Although not toxicity was observed in the *N. arenaceodentata* exposures with MI sediment, significant bioaccumulation and growth were observed in some treatments. Increased *N. arenaceodentata* growth (factor of ~1.5) was observed in MI contaminated sediment amended with chitin relative to both control and

unamended MI contaminated sediments, while apatite-amended sediment did not affect growth. These findings were similar to those observed previously with SD sediments. The increased growth with chitin was not surprising considering the added food source that was provided by both waterborne and sediment-dwelling bacteria (see Chapter 2), as well as the possibility of ingestion of the organic matter associated with the chitin itself. *N. arenaceodentata* build mucoid tubes in surficial sediment, protecting them from hypoxia, as well as providing a means for feeding on organisms at the sediment-water interface. Enhanced growth of *N. arenaceodentata* was also observed in acetate treatments. Although the increase was not statistically significant, this is once again likely due to feeding on the relatively high presence of bacteria in this treatment.

These results contrast with reduced *N. arenaceodentata* weight in exposures to activated carbon amendments, as observed by Millward et al. (2005). The authors suggested that ingested organic carbon likely reduced nutrient uptake due to its sorbent properties.

1.11.5.2. *N. arenaceodentata* body burden

While chitin resulted in increased *N. arenaceodentata* growth, whole body Cu, Zn, and Cd residues were significantly lower in the treatments that contained 0.5% chitin relative to the unamended MI sediment. Even at the lower dosing, chitin treatments resulted in marked bacterial blooms early on in the exposures. The reduced uptake of Cu, Zn, and Cd could be due to several reasons, including metal sorption to the chitin particles or dissolved organic carbon, the formation of insoluble metal-sulfides (or metal phosphate in the case of chitin and apatite combinations), and/or preferential feeding on the microorganisms inhabiting the overlying water column. Yang and Zall (1984) demonstrated effective sorption of Cu, Zn, Cd, Cr, and Pb to chitin, resulting in significant removal of these metals from aqueous solutions. In sediments, chitin has been shown to stimulate activity of sulfate reducing bacteria, resulting in metal-sulfides with low bioavailability (Robinson-Lora and Brennan 2009). While sulfides weren't quantified in these experiments, chitin and chitin/apatite mixed treatments possessed a particularly strong sulfide smell upon exposure breakdown.

Pore water metal concentrations (data not shown) did not show consistent trends and did not correspond with observed bioaccumulation differences. Lee et al (2000a, 2001) stressed that feeding and sediment ingestion, as opposed to pore water, may be the primary uptake pathway for metals for *N. arenaceodentata*. In addition, *N. arenaceodentata* may have to some extent avoided interaction with the interstitial water in favor of feeding on bacteria present in the overlying water.

In this study, there was no apparent reduction of Cu, Zn, or Cd tissue concentrations in apatite only treatments. It is possible that apatite concentrations greater than 5% might be required to reduce metal uptake by marine organisms from contaminated sediments. Although data for apatite were not found, Paller et al. (2009) demonstrated that organoclay concentrations of 50% significantly reduced uptake of PAHs in freshwater oligochaetes, while 15% formulations did not. The 5% dosing was selected for this study based on the approximate target doses that have been employed in pilot-scale studies where amendments have been tilled directly into the contaminated sediments (Millward et al. 2005, Cho et al. 2007). Further examination of the effectiveness of apatite to sequester cationic metals in marine sediments is therefore needed.

Cd body burdens were higher as a result of amendment with apatite, relative to unamended sediment. The concentration of Cd in the presence of apatite, however, was below that reported previously in control sediment (Moore and Dillon 1993). However, elevated uptake of Cd in the presence of apatite could be attributable to Cd impurities from the mined phosphate material (Knox et al. 2006).

1.12. Benefits

The goals of this work were to: 1) verify the overall conclusion of absence of toxicity associated with the amendments used in this project under more realistic exposure scenarios; and to 2) develop an exposure approach that could be used to increase realism for future assessments of amendment performance or the potential need to evaluate new amendments as the field evolves. This study demonstrated a lack of inherent lethal or sublethal toxicity associated with the inorganic amendments (North Carolina apatite and organoclay), yet some toxicity from organic amendments (chitin and acetate) that was likely associated with a microbial-induced decline in water quality. Evaluated sediment concentrations of 5% for apatite and organoclay, whether mixed directly in contaminated sediments or contained in geotextile reactive core mats, are therefore, not expected to pose an environmental risk to marine benthos. The presence of elevated ammonia and depressed dissolved oxygen concentrations in chitin and acetate treatments, respectively, are attributable to bacterially mediated processes. It is unclear from these laboratory experiments, however, as to how these organic amendments would react in actual marine systems versus the static systems used in this study. Therefore, it is advised that further investigation into the use of these materials as a means of *in situ* sediment management include the use of field studies.

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Chapter 2: Bacterial Community Response to Inorganic and Organic Amendments in Marine Sediments

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2.1. Abstract

Sediment amendments have been demonstrated promising strategies of enhancing removal of heavy metals and organic contaminants, where microbial activities play central roles in most remediation processes. The amendments apatite, organoclay (and apatite and organoclay in geotextile mats), acetate, and chitin were evaluated in uncontaminated sediments for impact on benthic community organisms (see Chapter 1), microbial community, and X-ray absorption spectroscopy. Significant bacterial biomass and activities were induced by amendments of apatite+ organoclay, chitin and acetate. Molecular fingerprints of bacterial communities by denaturant gradient gel electrophoresis (DGGE) showed that distinct bacterial populations occurred in overlying waters from different amendments: Apatite+ organoclay induced growth of *Gammaproteobacteria*, acetate enriched *Alphaproteobacteria* while *Bacteroidetes* and *Alphaproteobacteria* dominated in chitin treatment. In contrast, *Deltaproteobacteria* (*Desulfovibrio*), *Firmicutes*, *Bacteroidetes* were commonly found in the sediments amended with chitin, and apatite+ chitin. Sulfate-reducing bacteria (e.g., *Desulfovibrio*), and sulfide-producing/metal reducing bacteria were also recovered from most probable number (MPN) analyses in treatments with acetate, chitin, and apatite+ chitin. These geochemically important bacteria were stimulated by amendments and may play critical functional roles in the metal remediation process for future investigations of contaminated sediment.

2.2. Objective

The objective of this work was to assess the ecological impacts to benthic communities of technologies currently in field use at contaminated sediment including ...various amendments. The use of amendments in situ has been proved an effective approach to enhance removal of metal or organic contaminants under a variety of environments (Melton and Gardener, 2004; Brown et al., 2004; Werth et al., 2005; Anderson et al., 2003; Ishtok et al., 2004; Seager and Gardner, 2004). Currently, promising sediment amendments include inorganic (i.e., geotextile mats, apatite, organoclay, etc.) and organic materials (i.e., short chain fatty acids, chitin, etc.). Inorganic amendments such as phosphates (apatite) helped to immobilize several toxic metals (Cao et al., 2003; Melton and Gardener, 2004). Organoclays mediated in the binding of organic contaminants such as PCB's and PAH's (Mortland et al. 1986). In marine sediments, transitional metals formed relatively stable compounds via redox reactions (such as HS-) and/or were sequestered with phosphate, and thus the metals were no longer bioavailable (Brown et al., 2004). Organic amendments, in contrast, may induce indigenous microbes capable of bioreduction and/or of direct or indirect immobilization of toxic metals or other targeted contaminants. Several organic amendments have been field-tested to stimulate the indigenous microbial community to reduce or detoxify contaminated groundwater, aquifers, and sediments. For example, acetate or acetate plus other short chain fatty acids have been added as amendments for bioreduction of uranium (Anderson et al., 2003; Ishtok et al., 2004; Chang, 2005) and

selenate reduction (Lukas and Hollibaugh, 2001). In addition, the use of chitin and chitin with lactate has been applied for trichloroethylene degradation (Werth et al., 2005) and groundwater dechlorination (Vera et al., 2001). A secondary benefit of using organic amendments would be that once the microbial carbon and energy source is completely consumed, the bacteria that increased in numbers due to the presence of the added carbon source would decrease back to natural levels.

Microorganisms interact with metals in a variety of ways that lead to decreased metal solubility and mobility (Brierley, 1990; Tebo, 1995). Two biogeochemically important groups that have been shown to contain suitable physiology for metal precipitation and immobilization were iron-reducing and sulfide-producing bacteria (FeRB and SPB, respectively) due to their metabolic end-products, such as Fe(II) and HS⁻ (Lovley, 1993; Barnes et al., 1994; Barton and Tomei, 1995; Anderson and Lovley, 1997; Nealson, 1997; Tebo and Obraztsova, 1998; Arias and Tebo, 2003). Thus, in addition to strict inorganic chemical reactions, bacterially mediated remediation may play key roles in metal immobilization and metal remobilization.

Inorganic and organic amendments may improve the metal immobilization, transformation, and sequestration. However, what is much less understood and has not been thoroughly evaluated are the effects of the amendments on ambient living organisms including micro- and macro-organisms in marine environments. For example, little is known about the effect acetate and chitin have on microbial growth, and to our knowledge, no one has examined the population structure under these conditions. In this study, inorganic (geotextile mats, apatite, and organoclay) and organic (acetate, chitin) amendment combinations were applied to microcosms containing San Diego Bay and Mare Island Naval Shipyard sediments, and response of microbial communities and population dynamics (biomass and population structures) were monitored in both overlying waters and in sediments. Sulfate-reducing bacteria, and sulfide-producing/metal-reducing anaerobic bacterial groups were tested by most probable number (MPN) analysis with a variety of combinations of carbon sources and electron acceptors. In addition, X-ray absorption spectrometry was used on some of the sediment core samples to determine the effects of the amendments on the sediment composition. In parallel, ecotoxicological tests of exposure to amendments were conducted on survival of amphipods, survival and growth of polychaetes and larval fish as described in Chapter 1.

2.3. Technical Approach

Chapter 1 describes two different exposure series of the macro benthic response to different concentrations of amendments tested. The materials and methods used to perform the microbiology analysis in Exposure Series #1 and #2 are the same.

2.4. Experimental Design

The experimental design including sediment and amendments preparation are described in detail in Chapter 1. Briefly, apatite, organoclay, acetate, and chitin were used either singly or in combination as amendments. Amendments were either mixed directly into sediment or housed inside reactive core geotextile mats (Cetco®). Glass mason jars (1 L) were used as exposure vessels containing sediment samples and uncontaminated overlying filtered seawater. Amphipods, polychaetes, or larval fish were also added to the chambers for assessment of effects on macroorganisms. Beakers were continually aerated (~100 bubbles/minute) with filtered air.

Overlying water quality (pH, dissolved oxygen, ammonia, salinity, and temperature) were monitored daily. Experimental mesocosms were set up in replicates of five.

Based on the toxicity tests from the Exposure Series #1, dose-response experiments with acetate and chitin were followed (Section 1.5, Exposure Series #2), and microbial biomass was monitored on Day 0, Day 6 and Day 10.

In order to assess whether or not the amendments evaluated in clean sediment would also be non-toxic in field sediments contaminated with metals, similar exposures were conducted using a field-contaminated sediment. Course (reference sediment) from Yaquina Bay, OR (YBO) and fine-grained sediments and Mare Island Naval Shipyard (MINSY) were evaluated. MINSY sediments are known to be contaminated with heavy metals; whereas YBO sediments are considered clean and uncontaminated. Because the MINSY sediments were aged, additional spikes of Cu, Zn, and Cr were incorporated with the goal of metal concentrations in the overlying water of Cu= 0.25 ppm; and Zn and Cr=1.0 ppm. These concentrations had to assume no binding as TOC of coarse sand < 0.1%.

2.4.1. Sample Collection for Microbiology Studies

From each jar, approximately 100 mL of overlying water was collected aerobically without disturbing the sediment, and samples were stored in 50 mL centrifuge tubes at 4 °C. For sediments samples, three profiles were collected based on the horizontal depth: top layer, 0-1.0 cm deep; middle layer, 1.0-2.0 cm deep; and bottom layer, 2.0-3.0 cm deep. Sediment samples were collected and stored in 10 mL vials under anaerobic nitrogen conditions using nitrogen gas in the headspace.

2.4.2. Most Probable Number (MPN) Analysis

MPN analyses of overlying water and sediment horizon samples (top, middle, and bottom) were tested in anaerobically prepared test tubes or microtiter plates. Widdel's medium for marine sulfate-reducing bacteria was used. Acetate (20 mM), lactate (20 mM), and N-acetylglucosamine (20 mM) served as carbon sources (electron donors). Sodium sulfate (20mM) and sodium thiosulfate (20 mM) were used as terminal electron acceptors for sulfate-reducing bacteria and metal-reducing bacteria, respectively (Perry et al., 1993; Caccavo et al., 1996). The basic medium (per 1 Liter) contains 5 g NaCl, 0.4 g MgCl₂, 0.3 g KCl, 0.2 g KH₂PO₄, 0.15 g CaCl₂•2H₂O, 1 g yeast extract, 10 mL Vitamin solution (100X stock) (Kieft et al., 1999) and 10 mL Mineral solution (100X stock) (Bretschger et al., 2007).

2.4.3. Cell Number Determination by Epifluorescence Microscopy

Overlying water samples were collected, stained by SYBR Gold and observed following the protocol described by Chen et al. (2001) for counting microbial cells. Briefly, 0.5 mL water was fixed with 0.5 mL of 4% paraformaldehyde for 24 hours and filtered onto a 0.2 µm pore-size Al₂O₃ Anodisc 25 mm membrane filter (Whatman) with an approximately 10 kPa vacuum. The membranes were stained with 2.5XSYBR gold solution (final concentration) in the dark. The stained membrane filters were mounted on glass slides and covered with cover slips. The total bacterial cells were observed and counted under blue excitation (485nm) on a Zeiss Axioplan epifluorescence microscope (Zeiss, Germany) using 100 X Antiflex Neoflua oil objective lens.

2.4.4. DNA Extraction, PCR and DGGE

The overlying water samples were filtered by 0.2 μ filters (47 mm diameter) and DNA was extracted with lysozyme, Proteinase K, and SDS concomitant with phenol-chloroform extraction, and isopropanol precipitation as previously described (Kan et al., 2006). Three layers of sediment samples (0.3 g each) were extracted by UltraCleanTM Soil DNA Kit (MO BIO Laboratories) following the manufacturer's instructions. DNA concentration was estimated based on 260 nm absorbance using a Spectrophotometer ND-1000 (Nanodrop).

PCR amplification was performed in a 50 μ l reaction containing approximately 25 ng of template DNA, 25 μ l PCR Mastermix (Qiagen), 0.5 mM (each) primer, and water (double distilled). PCR program was performed with a Mastercycler gradient (Eppendorf). PCR primers used were 341f (GC) and 907r and the PCR program followed the protocol described by Scäfer and Muyzer (Scäfer and Muyzer 2001). Agarose gel electrophoresis was used to detect and estimate the PCR amplicons.

DGGE was performed as previously described (Kan et al., 2006), except the linear denaturant gradient was from 40 - 70% vice 40 - 65%. Briefly, DGGE was performed using a DcodeTM Universal Mutation Detection System (Bio-Rad) and similar concentrations of PCR products were loaded on a 1.5 mm-thick vertical polyacrylamide gel with a linear gradient of the denaturants urea and formamide. Electrophoresis was performed at 60 °C in 1 \times TAE buffer, and a voltage of 75V was applied for 16 hours. The DGGE gel was stained with SYBR Gold and photographed (Øvreås et al., 1997) with a CCD camera mounted on a UV transilluminator (UVP).

2.4.5. DGGE Band Sequencing and Phylogenetic Analysis

Representative bands were excised from DGGE gels and incubated in diffusion buffer (0.25 M ammonium acetate, 10 mM magnesium chloride and 0.1% SDS) at 50 °C for 30 minutes. One μ l supernatant was used to re-amplify the band. PCR products were purified by ExoSAP-IT (USB) and sequenced with primer 341f (no GC) by using Bigdye-terminator chemistry by ABI PRISM3100 Genetic Analyzer (Applied Biosystems).

All sequences were compared with GenBank database using BLAST, and the closest matched sequences were obtained and included in the downstream phylogenetic analysis. Phylogenetic trees were constructed using MacVector 10.0 software package (MacVector Inc.). Briefly, sequence alignment was performed with the program CLUSTAL W. Evolutionary distances were calculated using Jukes-Cantor method (Jukes and Cantor 1969) and distance trees were constructed using the neighbor-joining algorithm (Saitou and Nei 1987). Bootstrap values were obtained based on the analysis of 1000 re-sampling datasets.

Sequences of the partial 16S rRNA genes of representative DGGE bands have been deposited in the GenBank database under accession numbers GU938714-GU938760.

2.4.6. X-Ray Absorption Spectroscopy

Experiments were conducted at the Materials Research Collaborative Access Team's (MRCAT) beamline 10-ID, Sector 10 located at the Advanced Photon Source (APS), Argonne National Laboratory (ANL), Argonne, IL. The electron storage ring operated at 7 GeV in top-up mode. A liquid N₂ cooled double crystal Si(111) monochromator was used to select incident photon energies and a platinum-coated mirror was used for harmonic rejection. The beam energy

was calibrated by assigning the first derivative inflection point of the K_L-absorption edge of a zinc metal (9659 eV) foil. The samples were prepared as thin pellets with a hand operated IR pellet press and the samples were secured by Kapton tape. The zinc references were diluted with boron nitride to 1000 mg kg⁻¹ and formed into pellets. Reference materials examined include hopeite (Zn₃(PO₄)₂•4H₂O), Zn-Al layered double hydroxide with nitrate and silicate interlayers, Zn(OH)₂, ZnO, sphalerite (ZnS), zinc sorbed to ferrihydrite, zinc sulfate, aqueous zinc nitrate, franklinite (ZnFe₂O₄), willemite (Zn₂SiO₄), hemimorphite (Zn₄Si₂O₇(OH)₂•H₂O), and gahnite (ZnAl₂O₄). Five XAS spectra were collected in fluorescence mode at room temperature from -200 to 1000 eV relative to the absorption edge position of Zn with a Canberra multielement detector. The I₀ chamber was filled with N₂ while the I_L detector (for reference materials) contained approximately 60:40 Ar:N₂.

The collected spectra were analyzed using the Athena software program in the computer package IFEFFIT (Ravel and Newville, 2005) for data reduction and data fitting. The five individual spectra for each sample were averaged followed by subtraction of the background through the pre-edge region using the Autobk algorithm and normalized to an atomic absorption of one. The data were converted from energy to photoelectron momentum (k-space) and weighted by k³. Identification of zinc phases in the sediment samples was accomplished by principal component analysis (PCA) and linear combination fitting (LCF) of the sediment XAS spectra relative to the known reference spectra. PCA identified five suitable components for LCF validity.

2.5. Results

2.5.1. Exposure Series #1; Stimulation of Bacterial Growth and Microbial Biomass

Cell counts and fluorescence microscopy observations indicated that all the treatments, with the exception of those containing acetate, chitin, apatite + chitin, and apatite + organoclay did not change significantly over time (Figure 2-1A and Figure 2-1B). From initial point, the bacterial biomass seemed to become stable by day 10 in all the amendments, continuing through day 28 (Figure 2-1B).

The acetate treatment stimulated the largest bacterial bloom with the final cell density of $\sim 1.34 \times 10^8$ cells/mL, which was 3 orders magnitude higher than the control (2×10^5 cells/mL). Chitin only and apatite+chitin treatments reached the cell density of $\sim 7.56 \times 10^6$ cells/mL and $\sim 5.64 \times 10^6$ cells/mL, respectively (Figure 2-1A). The apatite + organoclay treatment also induced bacterial growth, with a final cell density of 1.76×10^6 cells/mL, slightly less than 10 times the control. In contrast, the cell numbers from other amendments remained similar to the controls, with a final cell density of $\sim 2.6 \times 10^5$ cells/mL.

In addition, different treatments stimulated distinct species of microbes based on the morphology as shown in Figure 2-1B. In the acetate treatment, most of the microorganisms were rod-shaped single cells, while in chitin and chitin + apatite treatments, multicellular filamental forms like trichomes were present (Figure 2-1B). One interesting difference was observed between chitin only and chitin + apatite treatments: quite straight multicellular filaments consisting of long-rod-shaped cells in chains were observed in chitin only but were not detected in chitin + apatite amended sediment.

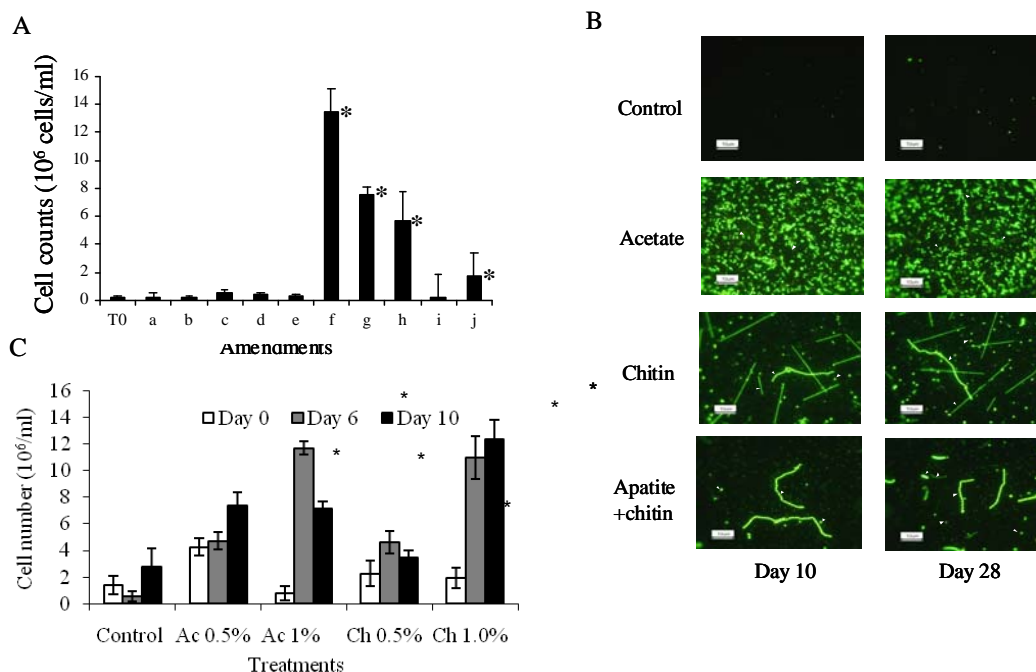


Figure 2-1. Microbial biomass during the amendment experiments. A, bacterial cell counts of overlying water. T0, control on day 0 and a-j, cell counts on day 28: a, control; b, mat only; c, mat+apatite; d, mat+apatite+organoclay; e, apatite only; f, acetate only (1:10 dilution); g, chitin only; h, apatite+chitin; i, organoclay only; j, apatite+organoclay. B, epifluorescence microscopic observation of planktonic bacteria with amendments of acetate, chitin, apatite and chitin on day 10 and day 28. Other amendment treatments showed similar results as control. C, bacterial cell counts for dose-response experiments. Ac, acetate; Ch, chitin. *, significant difference compared to the control (unpaired t-test, $p < 0.05$).

2.5.2. Exposure Series #2: Microbial Response in the Dose-Response Chitin and Acetate Toxicity Experiments

In dose-response experiments, both acetate and chitin significantly stimulated bacterial growth (Figure 2-1C). For acetate, 0.5% acetate did not change cell density significantly on day 6, but on day 10, the cell density increased from 4.27×10^6 cells/mL to 7.37×10^6 cells/mL ($P=0.05$). In addition, 1% acetate showed significant stimulation on both 6 days and 10 days, inducing the microbial density from 0.8×10^6 cells/mL to 1.1×10^7 cells/mL ($P<0.0001$) and 7.12×10^6 cells/mL respectively ($P<0.0001$). In contrast, 0.5% chitin treatment increased the cell density from 2.27×10^6 cells/mL to 4.59×10^6 cells/mL ($P=0.0014$), and the cell density decreased to 3.45×10^6 cells/mL on day 10 but still significantly high than the control ($P=0.0177$). Furthermore, 1% chitin increased cell density from 1.93×10^6 cells/mL to 1.10×10^7 and 1.23×10^7 cells/mL on day 6 and day 10 respectively ($P<0.0001$) (Figure 2-1C).

2.5.3. Exposure Series #3. Microbial Response in *N. arenaceodentata* Exposures with Field-Contaminated Sediments

In order to assess whether or not the amendments evaluated in clean sediment would also be non-toxic in field sediments contaminated with metals, similar exposures were conducted using a field-contaminated sediment. Sediment was collected from Green Sands Beach at Mare Island

Naval Shipyard (lat 38.086, long -122.255) and selected based on historically high bulk sediment metal concentrations (Table 1-2). The results were similar to results in the uncontaminated sediments. In the course grained sediments, the bacterial numbers were significantly higher by about 2-3 times; $2.0\text{--}3.5 \times 10^6$ cells/ mL in the samples containing the amendments chitin + apatite and chitin only (CACS and CCS) versus less than 0.5×10^6 cells/ mL in the sediments without chitin (CUU and CUS) (Figure 2-2). In the spiked, contaminated fine-grained sediments from Mare Island Naval Shipyard (all samples with the letter designation, 'F'), sediments with the chitin amendment had increased bacterial growth versus no chitin (Figure 2-2). Interestingly, the samples containing the spiked additional metals and chitin (FCS), were greater in numbers versus unspiked (FCU) at $\sim 2.5 \times 10^6$ versus 1×10^6 cells/ mL.

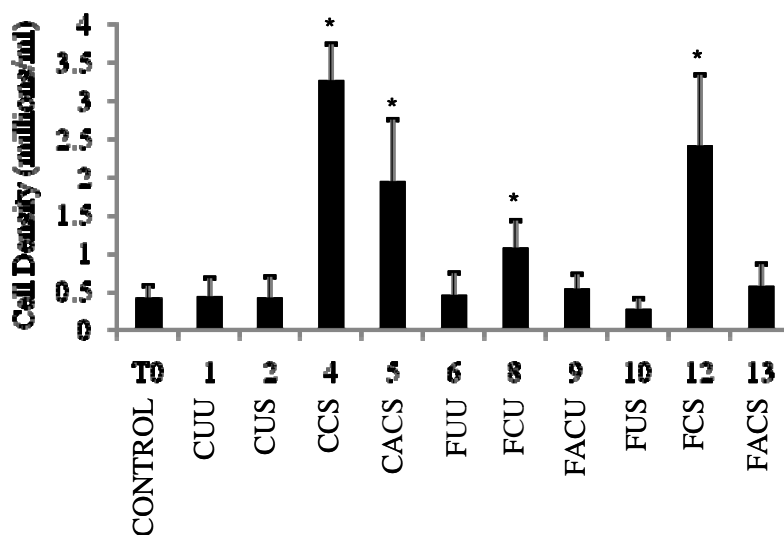


Figure 2-2. Cell density, 1×10^6 /mL, of bacteria in the overlying water column in course-grained and fine-grained sediments. Sediments were spiked with Cu, Zn, and Cr. Samples represent the control; CUU= Course- grained, Unamended, Un-spiked; CUS= Course- grained, Unamended, Spiked; CCS= Course-grained, Chitin amended, Spiked; CACS= Course- grained, Apatite + Chitin amended, Spiked; FUU= Fine-grained, Unamended, Un-spiked; FCU= Fine-grained, Chitin amended, Un-spiked; FACU= Fine-grained, Apatite + Chitin amended, Un-spiked; FUS= Fine-grained, Unamended, Spiked; FCS= Fine-grained, Chitin amended, Spiked; FACS= Fine-grained, Apatite + Chitin amended, Spiked.

2.5.4. Microbial Community in Overlying Waters

DGGE profiles of overlying water with various amendments showed that distinct bacterial populations were stimulated and dominated in most of the amendments (Figure 2-3); these data were not correlated with the cell numbers. In comparison with the control, the three mat samples, apatite and organoclay (b, e, and i in Figure 2-3) samples had quite similar patterns to the controls. In contrast, the acetate and chitin amendments (f and g in Figure 2-3) shifted the bacterial population structures significantly. For example, in the acetate treatment, two bands commonly found in controls, 16 and 17, disappeared while two new bands, 19 and 20, for an

uncultured Alphaproteobacterium became dominant. Meanwhile, bands 9, 10 and 11, became the dominant bands under the chitin-amended sediment (Figure 2-3). These bands represent Bacteroidetes and two unidentified Alphaproteobacteria, respectively. Under apatite and chitin conditions, the same 3 bands were dominant as under chitin only; the lower bands in the gel failed to yield sequence. Combined amendments had certain effects on bacterial communities and induced some bacterial groups to appear or disappear in corresponding treatments (c, d, h, and j in Figure 2-3).

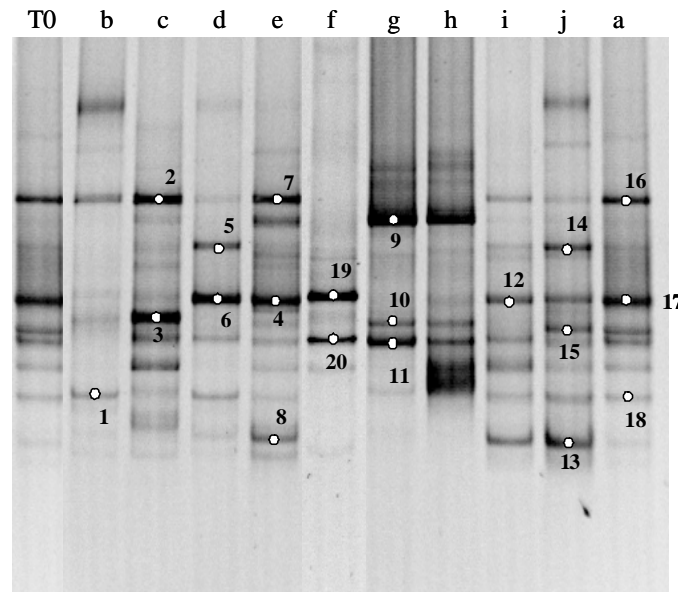


Figure 2-3. DGGE fingerprints of bacterial community from overlying waters on day 28. Labels a-j were the same as Figure 2-1. Bands 1-20 were selected and excised for sequencing.

Sequencing of the selected DGGE bands and phylogenetic reconstruction confirmed that amendments stimulated distinct bacterial groups in the treatments. The control treatments mainly contained bacterial groups of *Sulfitobacter* sp. (band 17) and *Ruegeria* sp. (bands 16 and 18,) (Figure 2-4). Besides the shared *Sulfitobacter* and *Ruegeria* groups, *Pseudoalteromonas* and *Halomonas* (Gammaproteobacteria) and *Croceibacter* (Bacteroidetes) were obtained in apatite+organoclay, and mat+apatite+organoclay amendments (bands 5, 13, 14, 15, Figure 2-3 and Figure 2-4). In contrast, acetate only amendment induced Alphaproteobacteria (Roseobacterial groups) (bands 19 and 20), while chitin and apatite+chitin stimulated *Phaeobacter*, *Roseobacter* (bands 10 and 11), and Bacteroidetes (band 9).

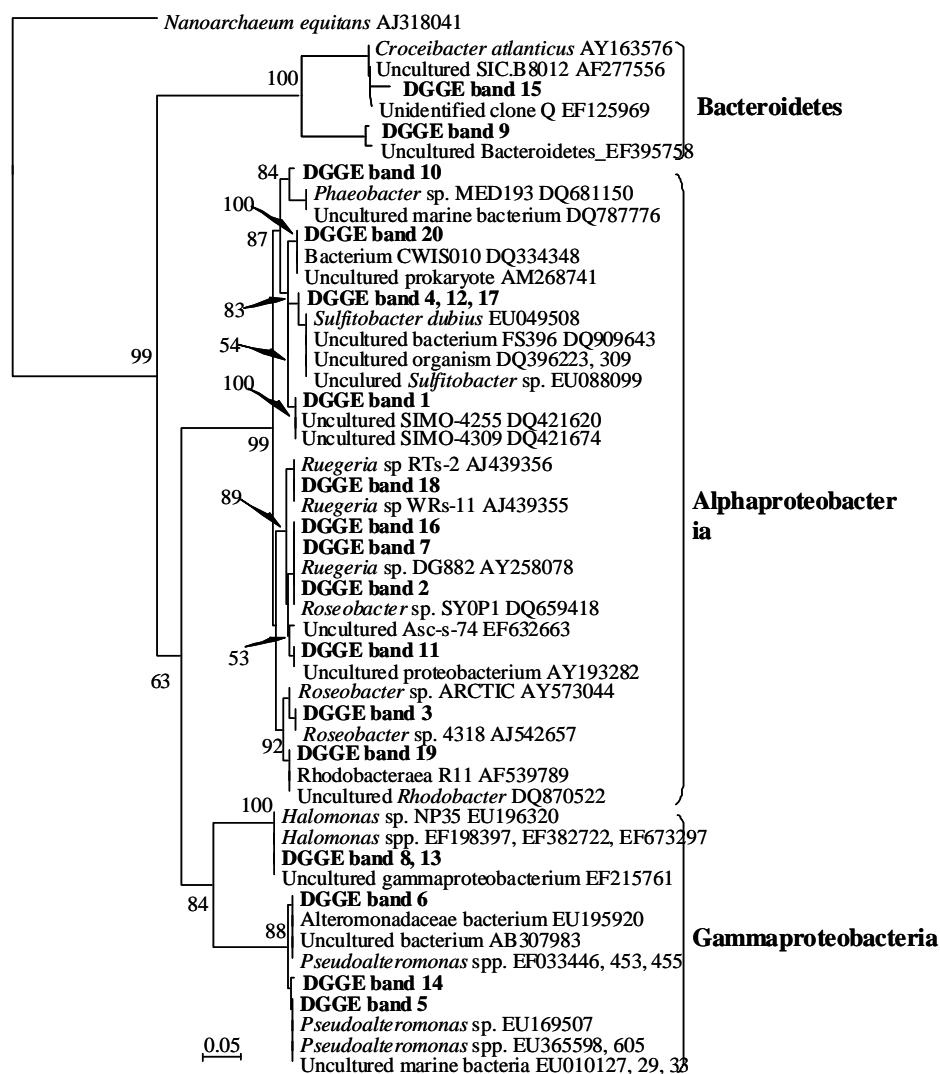


Figure 2-4. Phylogenetic analysis of DGGE band sequences obtained from Figure 2-3. Most closely related representatives from GenBank were included. Bootstrap values were calculated based on 1000 resampling datasets. For clarity, only bootstrap values relevant to the interpretation of groupings were shown. Scale bar indicated the number of substitutions per site.

2.5.5. Bacterial Communities in Sediments vs. Overlying Water

Due to the significant effects of amendments on microbial population structures within overlying water, we chose acetate only, chitin only, and apatite+chitin treatments to compare the bacterial communities between water and three sediment horizons. DGGE band patterns indicated that the stimulated bacteria in overlying water and in sediment were similar for acetate amendments (Figure 2-5, f), but were quite different for chitin, and apatite+chitin treatments (Figure 2-5, g and h). For example, Alphaproteobacteria and Bacteroidetes were dominant in overlying waters from chitin, and apatite+chitin. However, Deltaproteobacteria (*Desulfovibrio*, bands 25, 27, 37), Firmicutes (bands 21, 24, 33, 34, 36), and Bacteroidetes (bands 29, 30, 40, 41) were the primary retrieved phylotypes within the 3 sediment horizons analyzed (Figure 2-5, g and h, and Figure 2-6).

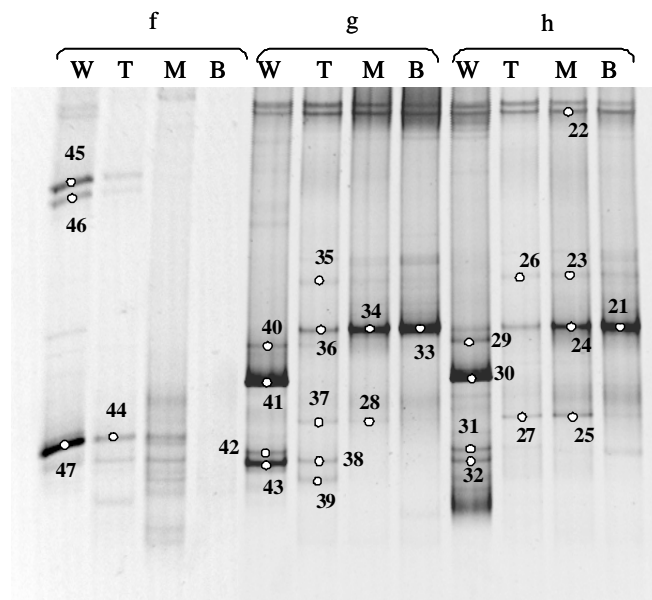


Figure 2-5. DGGE fingerprints of overlying waters and sediment bacterial communities exposed to acetate, chitin, and apatite+chitin collected on day 28. Amendments: f, acetate only; g, chitin only; h, apatite+chitin. Water samples (W) and three layers (T, top; M, middle and b, bottom) of sediments were included in the analysis. Bands 21-47 were excised for sequencing.

2.5.6. MPN Analysis

Compared to the control and other treatments, sulfate-reducing bacteria, and sulfide-producing/metal-reducing bacteria were significantly increased in all three horizons of sediments amended with acetate, chitin, and apatite+chitin. All three horizons of sediments showed positive results in MPN analysis (data not shown). In addition, sulfate-reducing bacteria and sulfide-producing/metal-reducing bacteria were also recovered in overlying water samples from the treatment with acetate, where became anaerobic (Chapter 1) during the experiments.

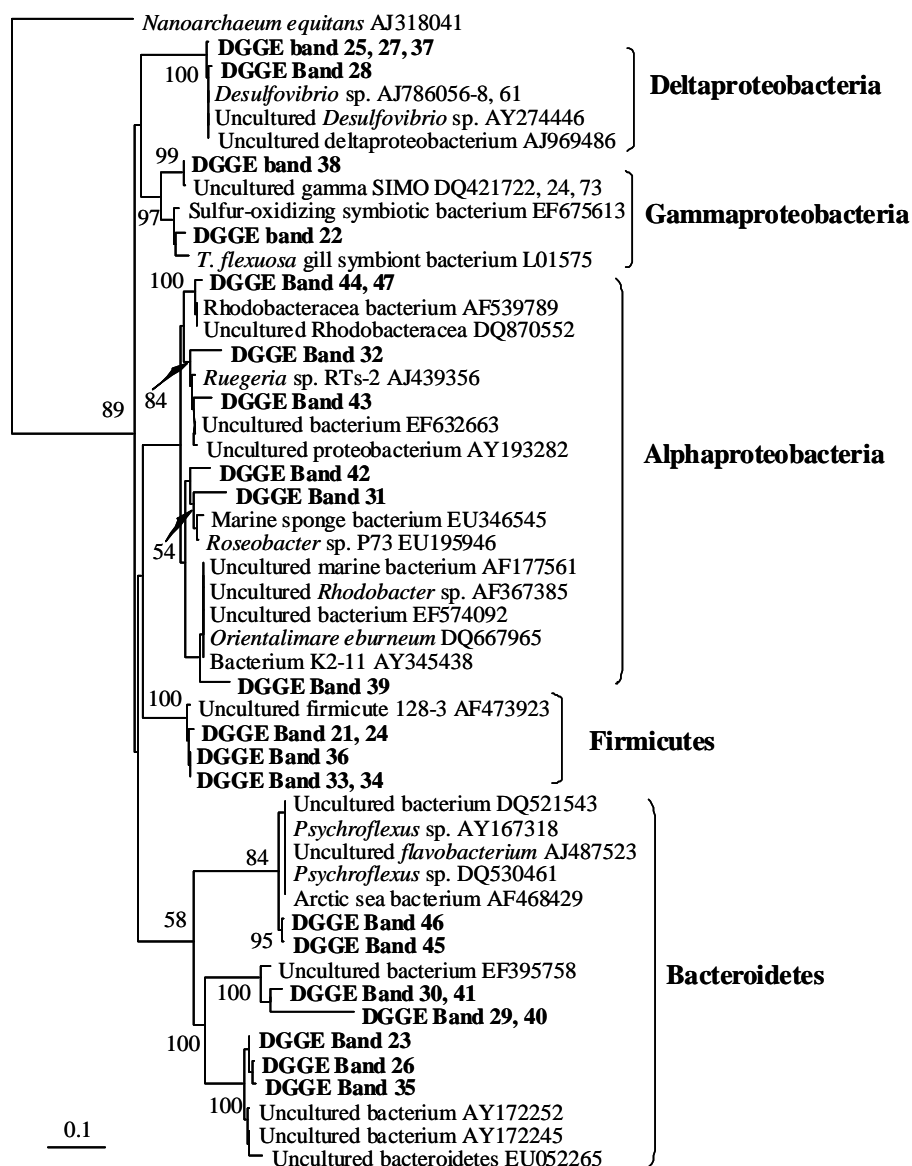


Figure 2-6. Phylogenetic analysis of DGGE band sequences obtained from Figure 2-5 and mostly closely related representatives from GenBank. Bootstrap values were calculated based on 1000 resampling datasets. For clarity, only bootstrap values relevant to the interpretation of groupings were shown. Scale bar indicated the number of substitutions per site.

2.5.7. XAS Analysis in Contaminated Sediments

The Zn XAS linear combination fitting results for two Mare Island (MI) Navy Shipyard samples amended with 5% apatite and followed over 120 days are shown in Table 2-1. Figure 2-7 shows an example X-ray absorption near-edge spectroscopy (XANES) spectra. Two samples (unamended and apatite amended) were analyzed by XAS at 28 days post apatite addition. The predominant initial Zn species is zinc hydroxide phases. At day 0, the MI3 sediment sample was identified to contain primarily easily mobile zinc phases; i.e., zinc hydroxide, smithsonite, Zn ferrihydrite, and Zn LDH silicate phases. By day 28 and 120, there is

a transition to hopeite, zinc phosphate, in the MI3 apatite amended samples. At day 0, the MI4 samples do contain some initial hopeite (~10%), but also show an increased transition to hopeite in the presence of the apatite amendment.

Table 2-1. Linear combination fitting of the samples from above as percent contribution for the mineral components. Hopeite is an independent component. Zn hydroxide-like phases falls into Zn hydroxide, Zn carbonate, and Zn LDH silicate. Sorbed Zn falls into Zn sorbed to ferrihydrite and gahnite. The R-factor is the error associated with the fits.

	Hopeite ($\text{Zn}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$)	Zn Hydroxide ($\text{Zn}(\text{OH})_2$)	Smithsonite (ZnCO_3)	Zn Sorbed to Ferrihydrite	Zn LDH Silicate	Gahnite (ZnAl_2O_4)	R-factor
MI3 0d	0.0	61.8	6.0	14.3	0.0	17.9	0.032
MI3 10d	0.0	61.3	5.6	14.8	0.0	18.3	0.033
MI3 28d	10.4	42.5	6.7	25.1	2.1	13.2	0.017
MI3 120d	32.4	22.0	3.3	34.9	0.0	7.3	0.004
MI4 0d	10.8	37.3	8.7	4.6	14.9	19.6	0.028
MI4 10d	12.3	37.7	10.1	2.1	19.3	18.6	0.026
MI4 28d	23.9	20.8	10.1	11.3	21.4	12.5	0.013
MI4 120d	34.2	11.4	5.4	24.9	17.2	7.0	0.003

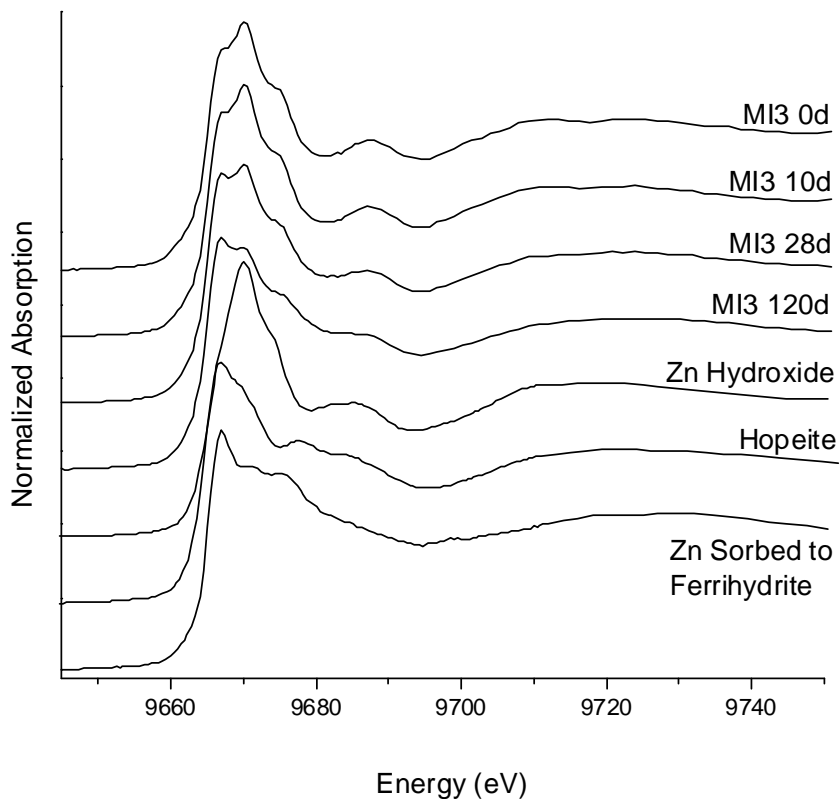


Figure 2-7. Example of XANES of Mare Island 3 samples over time post apatite amendment addition and reference spectra of primary components for linear combination fitting.

The Zn XAS linear combination fitting results of the spiked, contaminated MINSY sediments for the unamended and amended sediments (top 0.5 cm layer) are shown in Table 2-2. In general, these data are presented in a chart format, but for easier visualization in this report, they are displayed graphically in Figure 2-8. All are discrete samples 28 days post amendment(s) and were run in parallel to the 28 day toxicology experiment. The unamended, control sample showed that Zn was primarily in the easily mobile Zn hydroxide phase and demonstrated no significant changes to a more insoluble Zn phosphate. Due to very little instrument time, only the 10 day samples containing chitin were able to be run on the XAS. However, changes were observed in the presence of chitin and chitin + apatite versus the control sample. The most notable change in the chitin amended sample was the formation of ~13% hopeite (from zero) in the day 10 sample and a decrease in the Zn hydroxide phase (from ~65% to 55%) (Table 2-2 and Figure 2-8). In the apatite + chitin, hopeite was observed at day 0 post mixing (~10%), and increased to 27% by Day 10 (Figure 2-8 and Table 2-2).

Table 2-2. Linear combination fitting of spiked (Cu, Zn, Cr) fine-grained sediments (Mare Island Naval Shipyard) with no amendments, 0.5% chitin amendment, 5% apatite amendment, and mixtures of chitin and apatite (0.5% and 5% respectively) amendments. These are the percent contributions for the mineral components. Hopeite is an independent component. Zn hydroxide-like phases falls into Zn hydroxide, Zn carbonate, and Zn LDH silicate. Sorbed Zn falls into Zn sorbed to ferrihydrite and goethite. The R-factor is the error associated with the fits.

Sample	Reaction Time	Contribution Percentage				
		Zn Hydroxide ¹	Zn Carbonate	Sorbed Zn ²	ZnFe ₂ O ₄	Hopeite ³
Unamended	0 days	71	10	19		
	10 days	70	10	18		
	28 days	70		30		
Chitin Amended	0 days	63	25		13	
	10 days	55	17	5	11	13
Apatite Amended	0 days	45	16		14	25
	10 days	43	14		15	28
	28 days	31	9	26		34
Chitin + Apatite Amended	0 days	68	13		10	9
	10 days	52	15		8	27

¹ Includes Zn(OH)₂ and related layered double hydroxides

² Zn sorbed to an iron oxide

³ Zn₃(PO₄)₂

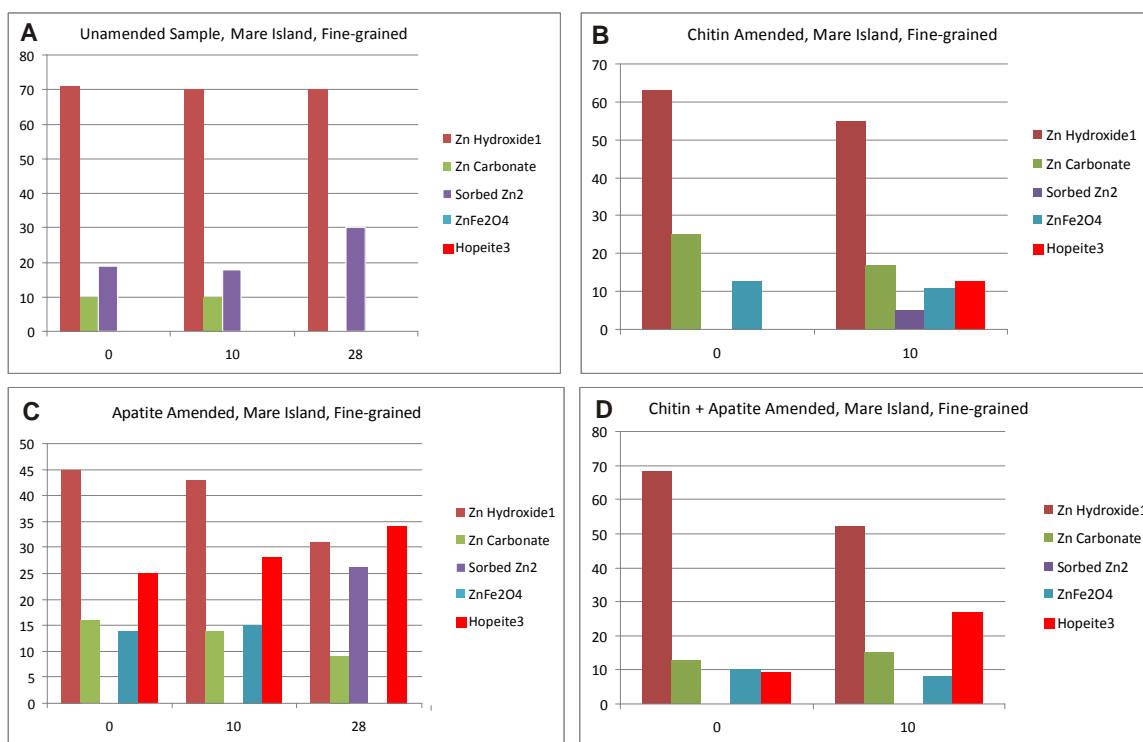


Figure 2-8. Data from Table 2-2, represented graphically. Fine-grained sediments (MINSY) were spiked with additional Cu, Zn, and Cr. A: Unamended, control; B: Chitin (0.5%) amendment; C: Apatite (5%) amendment; D: Chitin (0.5%) and apatite (5%) amendment.

2.6. Discussion

2.6.1. Effects of Inorganic Amendments on Bacterial Communities

Inorganic amendments applied alone in this study did not show significant effects. For example, the mat only, dispersed organoclay and dispersed organoclay + apatite were not significantly different from the control samples. Worthy of note, the inorganic amendments of mat material containing apatite versus dispersed apatite had a few distinct differences. The mat + apatite and apatite only had a common dominant band of *Rugeria* sp. (Figure 2-3, c and e, bands 2 and 7) observed in the control. However, the mat + apatite contained a *Roseobacter* (Figure 2-3, band 3) as a second dominant band relative to the *Sulfitobacter* (Figure 2-3, band 4) observed in the dispersed apatite. The mat + apatite + organoclay (Figure 2-3, d) differed in that the dominant band was *Pseudoalteromonas* spp. (bands 5 and 6). Different bacterial groups were observed in the water column, indicating that the amendments diffused out of the mat material and were capable of causing an effect on the bacteria in the water column.

The inorganic amendments did influence the microbial biomass or community structures when combined with other amendments. The chitin + apatite, and apatite + organoclay significantly increased the microbial biomass in the treatments (Figure 2-1A). In metal contaminated sediments, use of a mixed amendment would first allow the organic amendment (such as chitin) to stimulate the microorganisms that produce sulfides that quickly bind bioavailable metals. Under more oxic conditions, phosphate abiotically solubilized from the fine-grained fraction of the apatite amendment rapidly sequestered bioavailable metals from pore

waters to form metal phosphates, which was also observed by others (Ma et al., 1993; Laperche et al., 1996). Thus, efficient metal immobilization using phosphate relied on increasing the solubility of the phosphate. The mixed amendments might work together with the organic amendment promoting the growth of additional phosphate solubilizing bacteria, which in turn solubilizes more phosphate from the undissolved apatite fractions to sequester more bioavailable metals. Metal phosphates are typically more thermodynamically stable than the metal sulfides (Nriagu, 1974), so over time there should be a conversion of transitory metal sulfides to metal phosphates where these mixed amendment systems helped provide a more efficient and permanent metal sequestration.

Recent evidence suggests bacteria such as *Beggiatoa* sp. and *Thiomargarita* sp. may enhance phosphate gradients and promote the precipitation of apatite minerals (Schultz and Schultz, 2005). Furthermore, additions of organic materials (as carbon sources) enhanced the bacterial growth rate and phosphate solubilizing activities (De Souza et al., 2000). Bacterial involvement has been invoked to explain the formation of phosphorites (natural apatite mineral deposits) in both present day (Baturin, 1983) and ancient (Reimers et al., 1990; Leather, 1993) environments. In freshwater systems, we recognized the importance of phosphate solubilizing bacteria as they have been studied extensively for agricultural purposes (reviewed by Rodriguez and Fraga, 1999). Strains from genera *Bacillus*, *Rhizobium*, and *Pseudomonas* were some of the most prevailing phosphate solubilizers (Rodriguez and Fraga, 1999) and recently, diverse bacterial groups (i.e., *Serratia*, *Shewanella*, *E. coli*, *Vibrio* and *Proteus*) have been identified as capable of solubilizing generally considered insoluble phosphate compounds (Uzair and Amed, 2007) in soil systems. Some of these groups of bacteria (*Pseudomonas*, *Shewanella*, *Vibrio*) were common to ocean systems and therefore we expected they might also be capable of phosphate solubilization in marine systems. To date, very few studies have examined the microbial capacity for phosphate solubilization in marine systems (Ayyakkannu and Chandramohan 1971), except a recent study that demonstrated that specific carbon sources must be provided to encourage phosphate solubilization by attached and free-living marine bacteria (Uzair and Ahmed, 2007). In this study, attempts were made to isolate phosphate solubilizing bacteria (PSB) via standard methods for examination of (PSB). However, phosphate solubilizing bacteria would only solubilize apatite in culture medium for one transfer and solubilization of phosphate did not occur in subsequent culture transfers (Figure 2-2). Therefore, our ongoing and future work in the analysis of bacteria with capacity to solubilize rock apatite will fill this obvious gap and help us to fully understand the geochemical processes that will occur.

2.6.2. Effects of Organic Amendments on Bacterial Communities

Organic amendments such as acetate and chitin induced significant increase of bacterial cells and shifted the bacterial community composition of both water and sediments. Acetate is a two-carbon short chain fatty acid and serves a general food source for most of the microbes including bacteria, archaea and even eukaryotic microbes. The key enzymes (i.e., acetate kinase and phosphotransacetylase) of the acetate metabolic pathway are widely distributed in bacteria and thus acetate serves as the best carbon source to stimulate the growth of indigenous bacteria in natural environments (Ingram-Smith et al., 2006). In addition, chitin is an abundant structural polysaccharide produced by many marine organisms and it is a (1→4)- β -linked homopolymer of N-acetylglucosamine (NAG). The primary breakdown products of chitin are acetate and fructose, both of which are excellent carbon sources for anaerobic and facultative microorganisms capable of metal reduction (Bassler et al., 1991).

In the current study, amendments of acetate, chitin and chitin + apatite induced more diverse groups of *Roseobacter* in overlying waters, which were not dominant in the control (e.g., bands 3, 10, 11, 19, 20 in Figure 2-3 and Figure 2-4). In marine environments, *Roseobacter* was a major phylogenetic group (about 5-30% of total) and they were widely distributed across a wide gradient of environments (reviewed in Buchan et al., 2005). Members of *Roseobacter* have been found to be free-living, particle-associated, or in a symbiotic relationship with other living organisms (Buchan et al., 2005). One interesting physiological feature of *Roseobacter* was transformations in the biogeochemical cycling of sulfur. *Roseobacter* spp. harbored the ability to transform both organic (degradation) and inorganic (oxidation) forms of sulfur, including elemental sulfur, sulfide, sulfite and thiosulfate (Moran et al., 2003). Many metal ions react with different formats of sulfur (e.g., sulfide) and form relatively stable compounds via redox reactions (e.g., metal sulfides). Although little is known about the direct physiological roles of *Roseobacter* on heavy metals or organic contaminants in marine ecosystems, the fact that organic amendments stimulated the growth of *Roseobacter* suggests that the versatile physiological features of this group of Alphaproteobacteria deserves further study.

Organic amendments also stimulated distinct bacterial populations in sediments in comparison to overlying water. For instance, Deltaproteobacteria (*Desulfovibrio*), Firmicutes, and Bacteroidetes were dominate in the three horizons of chitin-amended sediments. *Desulfovibrio* is a genus of gram-negative sulfate-reducing bacteria (SRB) and *Desulfovibrio* species are commonly found in aquatic environments, usually with high contents of organic materials. SRB are anaerobic microorganisms that commonly used sulfate as the terminal electron acceptor. Besides high production of sulfide, SRB also displayed the capability of mediating electron flow and dissimilatory reduction of heavy metals, therefore, SRB could be beneficial in bioremediation of toxic metals via metal reduction and metal sequestration by HS⁻ (reviewed by Muyzer and Stams, 2008; Arias and Tebo, 2003; Barton and Fauque 2009). Firmicutes and Bacteroidetes are two common bacterial groups which are widely distributed in many environments, including soil, sediments, sea water and animal guts. To date, it is not clear if these two bacterial groups are centrally involved in metal bioremediation and this is partially attributed to the difficulty of cultivation of environmental microbes in the laboratory; despite our best attempts. For instance, our DGGE band sequences primarily matched with uncultured bacterial phylotypes in the GenBank (Figure 2-6). However, the high occurrence of these two groups of bacteria under heavy metal environments (Akob et al., 2006; Garau et al., 2007) suggests that 1) these two groups of microorganisms may be well adapted to the contamination sites, and 2) if not directly, these bacteria may cooperate with other microorganisms to facilitate the process of heavy metal immobilization or bioremediation in natural environments.

2.6.3. DGGE Analysis vs. MPN

Bacteria that play active roles in metal immobilization generally are facultative or anaerobic microorganisms and were usually in fewer numbers due to competition with other bacteria that might be tolerant of heavy metal contamination. These geochemically important bacterial groups were detected by MPN analysis (data not shown), which provides higher resolution when appropriate electron donors and acceptors are applied. In contrast, as a quick molecular fingerprint analysis, DGGE tended to bias towards detection of the dominant groups in the analyzed community (Muyzer et al., 1993; Kan et al., 2006). Thus, these geochemically important bacteria were likely not detected in the DGGE analysis except if they reached certain dominancy such as a group of sulfate-reducing bacteria (*Desulfovibrio*) in this study. Although

with a relative low abundance of biomass, these bacteria may play critical functional roles in the geochemical processes, including but not limit to metal bioremediation.

2.6.4. XAS Analysis

The Zn XAS linear combination fitting results for two Mare Island (MI) Navy Shipyard samples amended with 5% apatite and followed for 120 days demonstrates a significant reduction of zinc hydroxide phases and an increase in hopeite formation over time (Table 2-1). This is an important for two reasons. One, this demonstrates that similar to fresh-water systems, apatite does play a role in the transition of the heavy metal Zn from its more soluble form (hydroxides) to a more stable complex of hopeite (Zn phosphates). More interesting, this transition does not occur as quickly as in fresh-water systems. Therefore, this may be dependent on other processes such as bacterial re-mineralization of the apatite. Presence of phosphate-solubilizing bacteria was indicated (Figure 2-9), and they may have an effect on the phosphate re-mineralization. However, focused phosphate solubilizing studies need to be investigated to determine if bacteria do play a role in apatite solubilization.

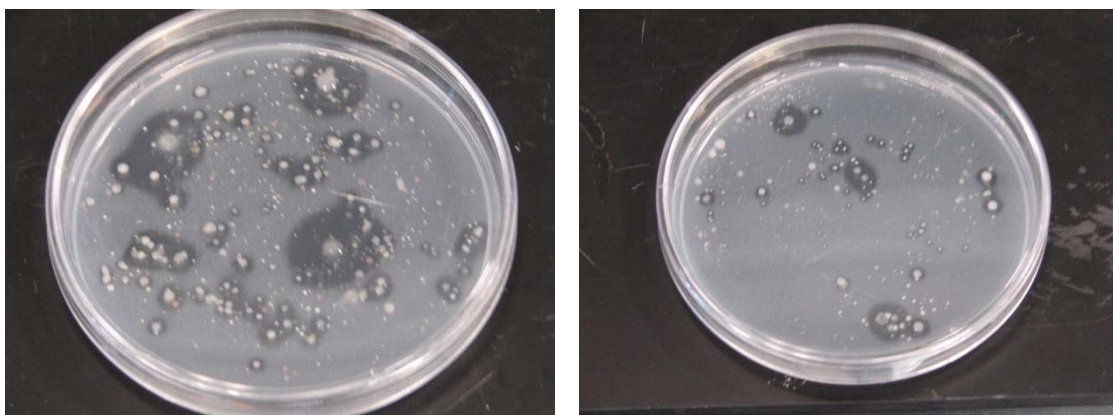


Figure 2-9. Bacteria plated onto medium containing insoluble apatite. Zones of clearing indicate phosphate-solubilizing bacteria (PSB).

The Zn XAS linear combination fitting results for the unamended and amended sediment samples (top 0.5 cm layer) are shown in Table 2-2. Two samples (unamended and apatite amended) were analyzed by XAS at 28 days post apatite addition. The predominant initial Zn species is zinc hydroxide phases. The 28 day unamended sediment sample was identified to contain about 70% easily mobile zinc phases; i.e., zinc hydroxide, smithsonite, Zn ferrihydrite, and Zn LDH silicate phases. The unamended samples demonstrated minor changes over the 28 day reaction period and no significant conversion to hopeite, the insoluble form of Zn. The most notable change in the chitin amended sample was the formation of some hopeite for the 10 day sample. The apatite amended sample observed very rapid changes with significant reduction in the quantity of zinc hydroxide and zinc carbonate phases and the formation of the zinc phosphate mineral, hopeite. The tandem amendment of chitin and apatite achieved a conversion of sediment zinc to hopeite in line with the rate of apatite alone after the 10 day reaction period. The apatite amended sample observed very rapid changes with significant reduction in the quantity of bioavailable zinc: zinc hydroxide and zinc carbonate phases. At Day 0, post the

addition of the amendments (apatite and chitin plus apatite), the zinc phosphate mineral, hopeite, was observed.

2.6.5. Microbiology, Water Quality and Toxicology

The above sediment amendments, especially those organic in nature, affected the survival or growth rates of various macro- and microorganisms under controlled laboratory exposure (Chapter 1). For example, amphipod and sheepshead minnow survival rates were significantly lower in acetate, chitin, and chitin + apatite treatments. Although chitin and apatite + chitin did not affect the survival rates for the sheepshead minnow and polychaete, the growth rates for the sheepshead minnow was significantly lower than the control, suggesting chronic toxicity; whereas growth rates in the polychaete were significantly higher under acetate, chitin, and apatite + chitin; suggesting the polychaete was able to feed off of the increased bacterial population (see Chapter 1). Reduced dissolved oxygen (acetate treatments) or excess ammonia (chitin treatments) were produced by microbial growth or microbial degradation of the amendments, such as the increased concentrations of ammonia under chitin conditions that aided in explaining the toxicological effects. It was likely the increased microbial biomass, shifted bacterial communities, and their induced microbial activities deteriorated the overlying water quality and subsequently affected the survival and growth rates of the macroorganisms present in this study. These results were corroborated by the Exposure Series #2 Dose-Response experiments. Meanwhile, we could not exclude the possibility that microbial pathogens were enriched by the amendments and directly infected the tested animals. However, this was out of the scope of current work and thus was not included in further discussion.

2.7. Benefits

In September 2004, SERDP held a sediments conference to outline the current state and future needs in sediment research (SERDP, 2004). There was agreement on the need to develop better *in situ* remedial options due to the high cost and environmental impacts of *ex-situ* options, such as dredging. These include the usage of amendments and the development of amendments/combinations of amendments for contaminant sequestration and the quantification of *in situ* microbial processes. This research demonstrated that microbiological data aided in the understanding of the macro benthic community toxicity effects (discussed in Chapter 1) observed. In addition, the geochemical analysis of the sediments aided in metal partitioning.

Sediment amendments demonstrated effects on ambient microbial assemblages. Treatments with apatite, organoclay, chitin and acetate induced significant increase of bacterial biomass and activities, and reshaped the microbial population structures. Distinct bacterial populations in overlying water and sediments were enriched by organic amendments including chitin and acetate. Due to the potential impact to water quality and living organisms, we recommend further efforts on investigating the effects of organic amendments and optimizing the concentration to apply in marine sediments. Due to the potential water quality and toxicology effects on the macro benthic communities (see Chapter 1), the tendency to be readily dissolved upon exposure to seawater, and cost effectiveness, acetate was not recommended for further investigation. In contrast, chitin, a naturally occurred structural polysaccharide in crustaceans, provides a potential for practical application in sediment amendments.

The utilization of X-ray Absorption Spectroscopy (XAS) was critical in the determination of how the heavy metal Zinc reacted to the presence of amendments. The data show that the 5%

apatite amendment will cause soluble Zn minerals to transition to hopeite, a stable mineral product that is not bioavailable. Chitin (0.5%) also aided in the transition of the bioavailable Zn spp to the non-bioavailable hopeite species. Chitin served as a food source to increase the normal bacterial flora in the samples, which in turn likely assisted in solubilizing naturally occurring insoluble inorganic phosphates. These inorganic phosphates likely then combined with the bioavailable Zn leading to the formation of hopeite. However, more research needs to be completed to fully address this question. In conclusion, incorporation of sediment amendments at select sites may be the preferred option of *in situ* remediation of heavy metal contaminated sediments.

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List of Scientific/Technical Publications

1. Articles for Peer- Reviewed Publication

- Kan, Jinjun, Yanbing Wang, Anna Obraztsova, Gunther Rosen, Jim Leather, Kirk Scheckel, Ken Nealson, and Y. Meriah Arias-Thode. *Manuscript in Preparation for Submission Aug 2010*. Bacterial Community Response to Inorganic and Organic Amendments in Marine Sediments.
- Rosen, Gunther, Jim Leather, and Y. Meriah Arias-Thode. *Manuscript in Preparation for Submission Aug 2010*. Ecotoxicological Response of Marine Organisms to Inorganic and Organic Sediment Amendments in Laboratory Exposure.

2. Technical Reports

None. Goal will be this report post acceptance by SERDP

3. Conference or symposium proceedings

- Arias, Y. Meriah, J. Kan, Y. Wang, G. Rosen, J. Leather, A. Obraztsova, K. Scheckel, and K. Nealson. Proceedings Paper Presentation. Bacterial and Benthic Community Response to Apatite, Acetate, and Chitin Amendments in Marine Sediment. Proceedings Paper Presentation. International Conference on the Biogeochemistry of Trace Elements. Chihuahua, Chihuahua, Mexico. July 13-16, 2009.
- Mathieu, J., John James, James Melhuish, Paula Mahoney, Y. Meriah Arias-Thode, M. Colosimo, and O. Peters. *Proceedings Paper Presentation: Multi-Scale Modeling of Bacterial Bioremediation*. American Society of Agricultural and Biological Engineers. Minneapolis, MN, June 2007.
- Scheckel, K., Y. Meriah Arias-Thode, J. Leather, and G. Rosen. Synchrotron Analysis of Metal Immobilization in Sediments. Proceedings Paper Presentation. International Conference on the Biogeochemistry of Trace Elements. Chihuahua, Chihuahua, Mexico. July 13-16, 2009.

4. Conference or symposium abstracts

- Arias-Thode, Y. Meriah, Gunther Rosen, Jim Leather, Jinjun Kan, Yanbing Wang, Anna Obraztsova, Kirk Scheckel and Kenneth H. Nealson. *Poster Presentation*. Bacterial and Benthic Community Response to Sediment Amendments. Strategic Enviro. Res. and Development Program. Washington, DC. Dec 2008.
- Arias-Thode, Y. Meriah, Gunther Rosen, Jinjun Kan, Anna Obraztsova, Jim Leather, Yanbing Wang, Kirk Scheckel and Kenneth H. Nealson. *Platform Presentation*. Benthic Community Response to Sediment Amendments. Society of Environmental Toxicology and Chemistry. Tampa, FL. Nov 2008.

- Arias-Thode, Y. Meriah, Gunther Rosen, Kirk Scheckel, Yanbing Wang, Anna Y. Obraztsova, Jim Leather, Jinjun Kan, and Kenneth H. Nealson. *Poster Presentation*. 257: Bacteria, Polychaete, & Geochemical Response to Apatite and Chitin in Metal Contaminated Sediments. Strategic Enviro. Res. and Development Program. Washington, DC. Dec 2009.
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- Arias-Thode, Y. Meriah. *Seminar Presentation*. 27 February 2008. "Bacterial and Benthic Community Response to Sediment Amendments" to SSC Pacific Code 7175 Seminar Series.
- Arias-Thode, Y., Gunther Rosen, Jinjun Wang, Anna Obraztsova, Jim Leather, Yanbing Wang, Kirk Scheckel, and Kenneth Nealson. *Platform Presentation*. Polychaete Response to Apatite and Chitin in Metals Contaminated Sediments. Association of Environmental Health and Sciences, San Diego, CA. Mar 2010 .
- Arias-Thode, Y., Gunther Rosen, Jinjun Wang, Anna Obraztsova, Jim Leather, Yanbing Wang, Kirk Scheckel, and Kenneth Nealson. *Platform Presentation*. Benthic Community Response to Sediment Amendments. Association of Environmental Health and Sciences, San Diego, CA. Mar 2009.
- Kan, Jinjun, Y. Meriah Arias-Thode, Yanbing Wang, Anna Obraztsova, Gunther Rosen, Jim Leather, and Kenneth H. Nealson. *Poster Presentation*. Bacterial and Benthic Community Response to Inorganic and Organic Sediment Amendments. International Society of Microbial Ecology. Cairns, Australia. Aug 2008.
- Kan, Jinjun. *Seminar Presentation*. 18 April 2008. "Bacterial and Benthic Community Response to Sediment Amendments" to Southern California Geobiology School of Engineering Seminar.

5. Text books or book chapters

None.